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## Canadian Journal of Zoology

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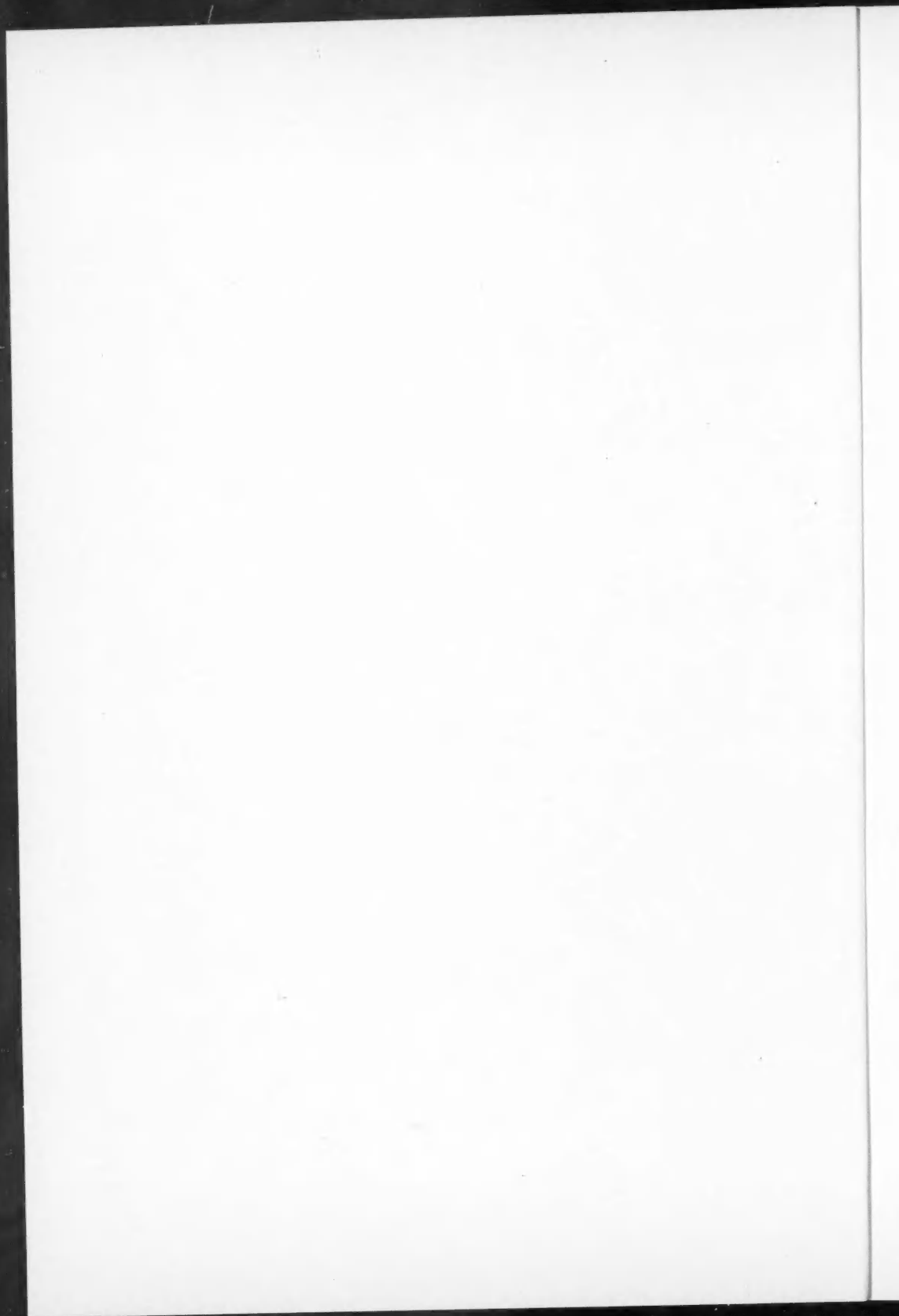
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## AN EXPERIMENTAL STUDY ON THE EFFECT OF THE SWIMBLADDER ON HEARING SENSITIVITY IN *AMEIURUS NEBULOSUS NEBULOSUS* (LESUEUR)<sup>1</sup>

H. KLEEREKOPER AND PETRONELLA A. ROGGENKAMP

### Abstract

Hearing sensitivity was observed in *Ameiurus nebulosus nebulosus* (LeSueur). A threshold curve over the frequency range from 210 to 1840 c.p.s. was established for normal fish as well as for fish in which the swimbladder had been mutilated. A comparison of both threshold curves indicates that the sensitivity in operated fish was decreased in the whole frequency range: for the frequencies from 330 to 750 c.p.s. by about 13 db; for the frequencies of 1000, 1300, and 1500 c.p.s. by respectively 23, 27, and 30 db. The stronger decrease at the higher frequencies was attributed to the loss or modification of the resonance of the opened swimbladder. The sensitivity of the operated fish increased in the frequency range from 330 to 210 c.p.s. This increase in sensitivity is abolished when the lateralis branch of the vagus is cut and must therefore be attributed to perception of low frequency vibrations by the lateral line.

### Introduction

Although numerous investigators observed hearing in fish or studied the organs involved in the perception of vibrations, only a few of them made direct observations on the acoustical function of the swimbladder or investigated the effect of the resonance of the bladder on hearing sensitivity in fish.

Weber (16) was the first to suggest that the function of the swimbladder was related to hearing by transmitting sound vibrations from the water to the Weberian ossicles. This view was criticized especially by Hasse (11), Bridge and Haddon (3), and Thilo (15). However, the anatomical studies on the inner ear, and the relationships between the Weberian ossicles and the swimbladder by de Burlet (4), Evans (5, 6, 7), Wohlfahrt (17, 18), and von Boutteville (2) lent support to Weber's theory. Their conclusions were supported by the experiments of von Frisch (8, 9) and von Frisch and Stetter (10).

No work was done on the acoustical function of the swimbladder during the following years until Poggendorf (14) investigated the absolute threshold curve in *Ameiurus nebulosus* and made further observations on the Weberian apparatus in Ostariophysi. That author was not able to establish the acoustical function of the swimbladder in catfish but determined the resonant

<sup>1</sup>Manuscript received June 23, 1958.

Contribution from the Department of Biology, Hamilton College, McMaster University, Hamilton, Ontario.

frequency of an isolated swimbladder of *Phoxinus laevis* by submitting it to sound when submerged in water. See also Autrum and Poggendorf (1).

In observations on the threshold curve of hearing as a part of their experimental studies on the cyprinid *Semotilus atromaculatus atromaculatus*, Kleerekoper and Chagnon (12) found that in this species the sensitivity increased between 800 and 2400 c.p.s. Although they put these findings down to inaccuracies of their observations, similar experiments on hearing perception (unpublished results) revealed a corresponding dip in the threshold curves of the cyprinids *Rhinichthys*, *Notropis*, and *Chrosomus*. It was believed that this phenomenon was brought about by the cavity resonance of the gas in the swimbladder.

In order to further investigate this phenomenon a comparison of the auditory threshold curves in normal fish and in fish in which the swimbladder resonance had been abolished was made.

### Materials and Technique

*Ameiurus nebulosus nebulosus* (LeSueur) was selected as a species which is easily trained to associate sound with food and which is highly resistant to surgical intervention.

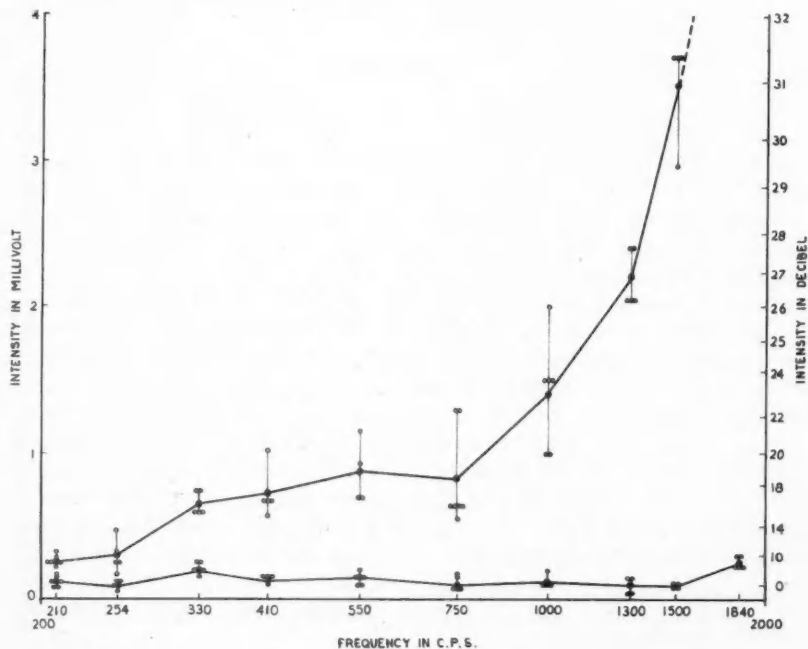


FIG. 1. Threshold curves for hearing in *Ameiurus nebulosus nebulosus*. Lower curve: normal animal. Upper curve: after deflation of the swimbladder by mutilation. Fish No. I.

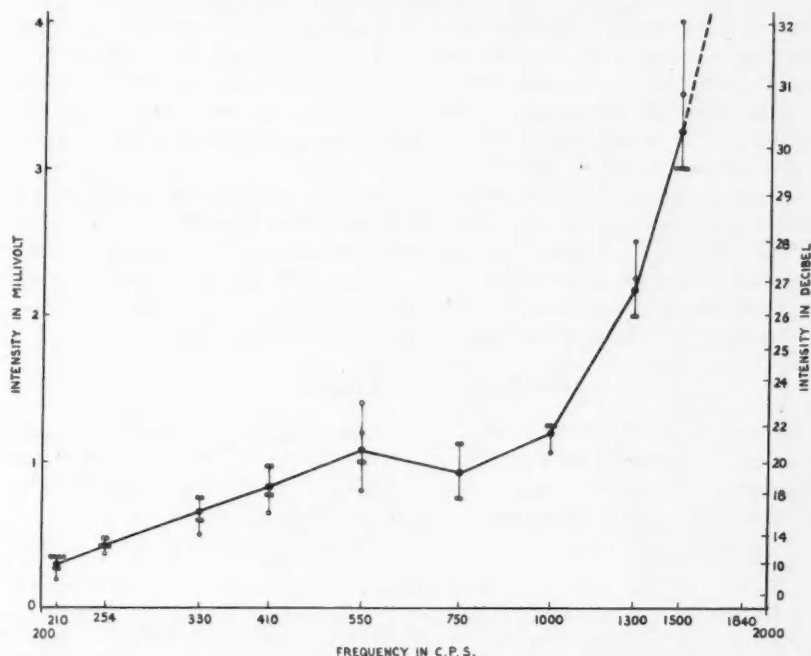


FIG. 2. Threshold curve for hearing in *Ameiurus nebulosus nebulosus* after deflation of the swimbladder by mutilation. Fish No. II.

For these experiments a low frequency oscillator with a continuously variable frequency range of 5000 to 6000 c.p.s. served as a source of sound, transmitted by electromagnetic speakers. The output of the oscillator could be regulated.

Sound intensities in the water were measured with a crystal hydrophone and a standing wavemeter as described by Kleerekoper and Chagnon (12). The output of the oscillator was calibrated against the corresponding values of sound intensities expressed in millivolts and decibels as measured by the standing wavemeter for the frequencies used in these experiments.

The experiments were carried out in aquaria (75 × 30 × 35 cm). A screen divided each aquarium broadwise into two equal sections. The fish was kept in one section and in the other the speaker was suspended. At the front side a wooden shield was placed to prevent the fish from seeing the movements of the experimenter. Small wide-angle lenses placed in the shield allowed the experimenter to observe the whole section in which the fish was kept.

### Surgical Operations

In *Ameiurus* the lateral walls of the swimbladder are in close contact with the inner surfaces of the skin. To eliminate the resonance of the bladder its lateral wall was opened and a piece cut out of such a size (about 1 cm in

diameter) that healing would not occur in the course of the experiment. Since the anterior dorsal wall is attached to the vertebral column, the whole bladder could not be removed without serious injury to the animal. Although the swimbladder did not collapse, it was deflated by the above operation; the decrease of its volume was visible externally by the compression of the body wall posteriad to the pectoral fins.

In one experiment the function of the lateral line was impeded by removing a length of the lateralis branch of the vagus nerve near the gill clefts.

The behavior of the fish was not greatly altered by the operation. Their locomotion was somewhat labored as a result of the loss of hydrostatic pressure and they remained resting on the bottom of the aquarium most of the time.

One per cent solution of urethane was used to anaesthetize the fish.

### Experimental Work

Normal fish and fish in which the swimbladder had been mutilated were trained to associate sound of 750 c.p.s. with the supply of food. After conditioning, the lower and upper limit of hearing in the fish were established by gradually decreasing or increasing the frequency of the sound.

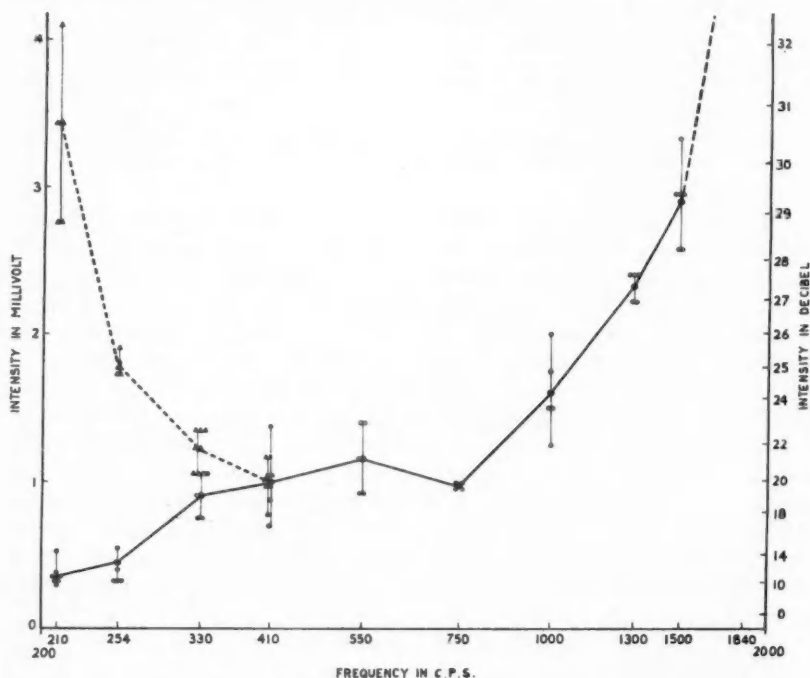


FIG. 3. Threshold curves for hearing in *Ameiurus nebulosus nebulosus* after deflation of the swimbladder by mutilation. Solid line: lateralis branches of the vagus intact. Broken line: after section of the branches. Fish No. III.

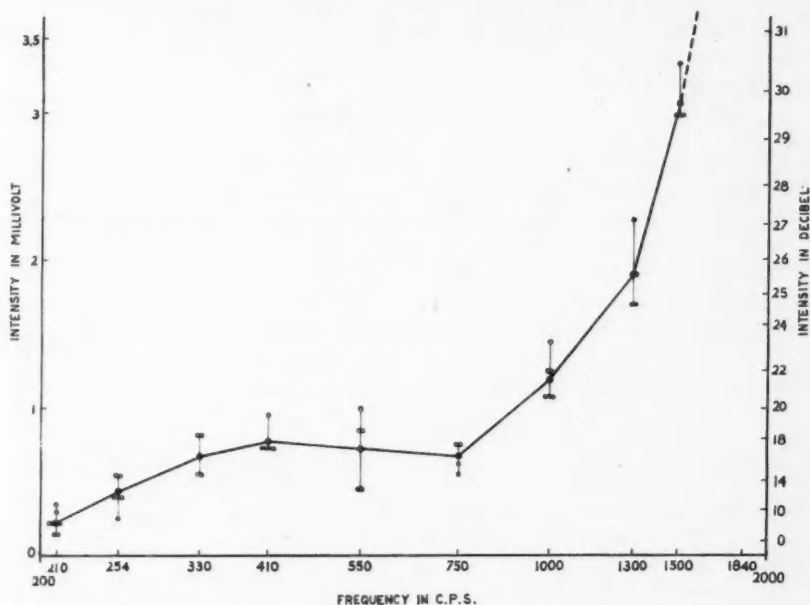


FIG. 4. Threshold curve for hearing in *Ameiurus nebulosus nebulosus* after deflation of the swimbladder by mutilation. Fish No. IV.

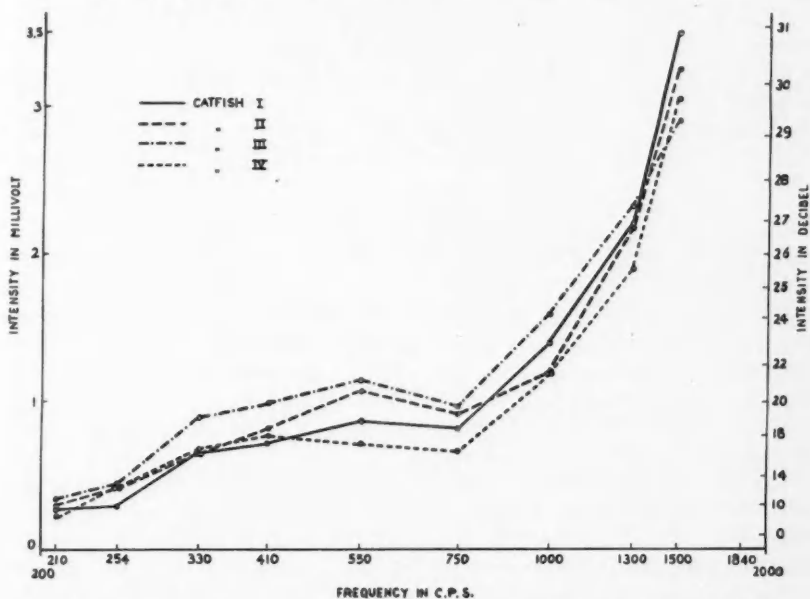


FIG. 5. Threshold curves for hearing in fishes I to IV after deflation of the swimbladder by mutilation.

The threshold curve of hearing was determined by measuring the threshold values for the frequencies of 750, 1000, 1300, 1500, 1840, 550, 410, 330, 254, and 210 c.p.s.

### Experimental Results

The frequency range perceived by normal catfish was from 30 c.p.s. to over 6000 c.p.s.; in operated fish these values were 50 c.p.s. and 4500 c.p.s.

For all the frequencies tested, the experimentally established threshold values were plotted against the frequency on the logarithmic scale. The threshold curves of the four operated fishes are represented respectively in Figs. 1, 2, 3 (solid line), and 4; that of the normal fish in Fig. 1 (lower curve).

The values in decibels of the threshold curves of the four operated specimens (I, II, III, and IV) are compared with the values of the average threshold curve of normal catfish (according to Poggendorf (14)) in the following table:

TABLE I

The threshold values for hearing in *Ameiurus nebulosus nebulosus*, in decibels, in normal individuals and in those with mutilated swimbladder

Frequency, in c.p.s.	Operated catfish					Normal catfish, av.
	I	II	III	IV	Av.	
210	9	10	11	7	9	5
254	10	12	13	12	12	6
330	16	16	16	19	17	7
410	17	18	20	19	18	7
550	19	21	21	17	20	5
750	18	19	20	18	19	0
1000	23	22	24	22	23	0
1300	27	27	28	25	27	0
1500	31	30	29	30	30	0
1840	35	35	35	35	35	—

### Discussion

The threshold curves for all the operated fish had approximately the same shape for the frequency range of 210 to 1840 c.p.s. with individual differences of 0 to 4 db (see Table I and Fig. 5).

The threshold curves of fish I before and after mutilation are compared in Fig. 1 (respectively lower and upper curves). The loss of sensitivity covers the whole frequency range from 210 to 1840 c.p.s. The striking decrease in sensitivity (in operated fish) in the frequency range from 750 to 1840 c.p.s. resulted from the decrease or absence of resonance in the opened swimbladder (see also Figs. 2, 3 (solid line), and 4).

The same phenomenon is demonstrated in Fig. 6, in which the average absolute threshold curve of the four operated catfishes is compared with the absolute auditory threshold curve for catfish (*Ameiurus nebulosus*), as established by Poggendorf (14).

The increase of sensitivity in the range from 330 to 210 c.p.s. results probably from the perception of vibrations of low frequency by the lateral line.

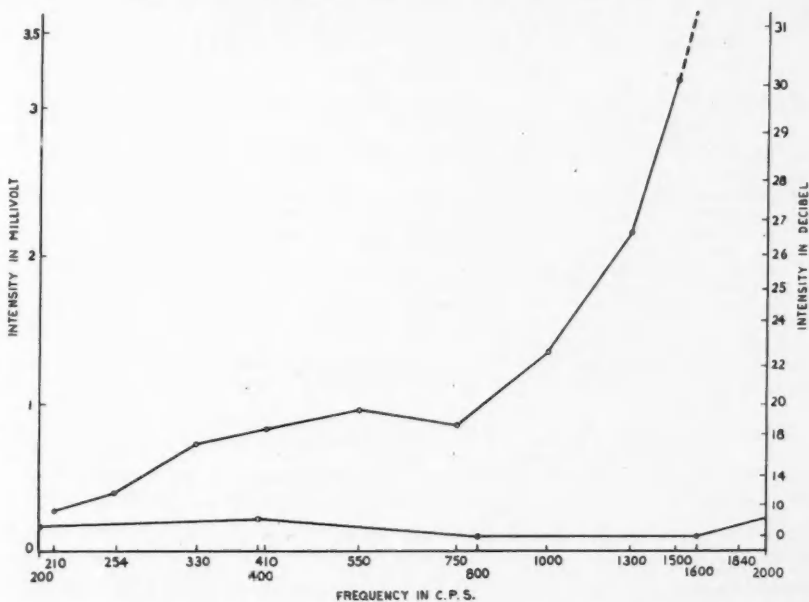


FIG. 6. Threshold curves for hearing in *Ameiurus nebulosus nebulosus*. Lower curve: normal fish according to Poggendorf (14). Upper curve: curve compiling all data of fishes Nos. I-IV.

This was investigated in one of the catfish in which the lateral line nerve was at first left intact (Fig. 3, solid line) and later cut at both sides (Fig. 3, broken line). Observations by Parker and van Heusen (13) also proved that the lateral line organs of *Ameiurus nebulosus* are stimulated by low tones of an underwater source (43 to 344 c.p.s.) but not by higher tones (344 to 2752 c.p.s.).

### Conclusion

The acuity of hearing in *Ameiurus nebulosus nebulosus* (LeSueur) is increased by the swimbladder in the whole frequency range of perception but particularly at the resonant frequency of the bladder. The lateral line organs aid in the perception of low frequency vibrations.

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## THE NORMAL BLOOD CHEMISTRY OF THE BEAVER (CASTOR CANADENSIS)

### B. BLOOD GLUCOSE, TOTAL PROTEIN, ALBUMIN, GLOBULIN, FIBRINOGEN, NON-PROTEIN NITROGEN, AMINO ACID NITROGEN, CREATINE, CREATININE, CHOLESTEROL, AND VOLATILE FATTY ACIDS<sup>1</sup>

A. B. STEVENSON,<sup>2</sup> W. D. KITTS,<sup>3</sup> A. J. WOOD,<sup>3</sup> AND I. MCT. COWAN<sup>4</sup>

#### Abstract

Blood glucose, total protein, albumin, globulin, fibrinogen, non-protein nitrogen, amino acid nitrogen, creatine, creatinine, cholesterol, and volatile fatty acids have been determined on blood samples taken from yearling and adult beavers. In most cases the values of the summer determinations are much lower than those taken in the fall for the two age classes under study. Where different, the blood values of adult beaver are higher than those of yearlings.

#### Introduction

The beaver is an aquatic rodent including in its diet large quantities of woody material and could therefore show certain metabolic peculiarities when compared with the common laboratory rodents. The adequate interpretation of the nutritional studies we have in progress on the beaver require that the concentration of the major blood components of normal animals be established. The normal values for packed-cell volume, sedimentation rate, hemoglobin, erythrocyte diameter, and blood cell counts have already been presented (6).

#### Methods and Materials

##### (a) *Experimental Animals*

A description of the experimental animals, their dietary regimen, housing, and the methods used in the procurement of the blood samples will be presented elsewhere.

##### (b) *Analytical Procedure*

(i) Blood sugar determinations were carried out using the titration method of Shaffer and Hartmann and Somogyi (7). Where possible the tests were performed within 2 hours from the time the blood was obtained. During this interval the samples were refrigerated to minimize autolytic changes.

(ii) Total protein in the whole blood, plasma, and serum; albumin; globulin; and fibrinogen were determined by the micro-Kjeldahl method outlined by Steyermark (11) and Hawk *et al.* (5).

(iii) Non-protein nitrogen (N.P.N.) was determined by the modified Folin-Wu method (7) and also by the procedure given by Steyermark (11).

<sup>1</sup>Manuscript received October 6, 1958.

Contribution from the Department of Zoology and the Division of Animal Science, The University of British Columbia, Vancouver, British Columbia. Financial assistance was received from the National Research Council of Canada.

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<sup>3</sup>Division of Animal Science, The University of British Columbia.

<sup>4</sup>Department of Zoology, The University of British Columbia.

(iv) Amino nitrogen was determined colorimetrically by the modified Danielson method (5).

(v) Creatine and creatinine were determined according to the method outlined by Folin-Wu (5).

(vi) Cholesterol was determined by the modified Bloor-Sackett method as outlined by Koch and Hanke (7).

(vii) Volatile fatty acids were determined by the method outlined by Neish (9), without modification.

## Results and Discussion

### (a) Total Reducing Sugars

As indicated by the wide range and the large standard deviation recorded, there was considerable variation in the total reducing sugars. There was no significant difference between the yearling and adult values (Table I). The data given suggest that the total blood sugar level in normal beaver is comparable to that for the other monogastric animals and for the suckling young ruminant (1).

TABLE I  
Some normal blood values for the beaver (*Castor canadensis*)

	Yearling beaver			Adult beaver		
	Mean and standard error	Standard deviation	Range	Mean and standard error	Standard deviation	Range
Blood sugar, mg %	95.8 $\pm$ 5.2	27.0	67 - 160	98.8 $\pm$ 5.7	26.6	49 - 141
Total protein (whole blood), g %						
A. Summer level	17.72 $\pm$ 0.33**	1.00	16.49-19.10	19.43 $\pm$ 0.23**	0.72	18.14-20.18
B. Autumn level	20.03 $\pm$ 0.41*	1.37	19.18-23.55	20.40 $\pm$ 0.58	1.54	17.99-22.31
C. Total	18.85 $\pm$ 0.36	1.67	16.49-23.35	19.40 $\pm$ 0.29	1.38	16.89-22.31
Total protein (serum), g %						
A. Summer level	6.70 $\pm$ 0.10**	0.32	6.08- 7.04	7.61 $\pm$ 0.17**	0.54	6.81- 8.69
B. Autumn level	7.28 $\pm$ 0.07*	0.23	7.06- 7.71	7.63 $\pm$ 0.25	0.67	6.46- 8.44
C. Total	6.96 $\pm$ 0.04***	0.19	6.08- 7.71	7.49 $\pm$ 0.14***	0.62	6.46- 8.69
Total protein (plasma), g %						
A. Summer level	6.87 $\pm$ 0.12	0.27	6.39- 7.04	8.24 $\pm$ 0.22	0.57	7.61- 9.08

\*, \*\*, and \*\*\* indicate comparisons significant to the .01 level.

### (b) Total Protein

Total protein in whole blood includes albumin, globulin, fibrinogen, hemoglobin, and, to a lesser degree, the proteinaceous material of the blood cell walls. Hemoglobin is the major constituent and therefore any variation in it will be quickly reflected in the total protein level.

The total proteins of the whole blood for both age groups are shown (Table I) to be significantly lower in the summer than in the autumn. It has already been shown (6) that the hemoglobin level of both age groups is significantly lower in the summer than in the autumn. The seasonal change in the hemoglobin accounts for only about half of the change in total protein between summer and autumn. This suggests that the increase involves protein

constituents other than the hemoglobin fraction. In view of the concentration of growth into the summer period in the wild mammals in the northern hemisphere (3), it can be suggested that the lowered total protein levels that we have noted in the summer in deer and in beaver reflect the seasonal demand for amino acids to be used for the repletion of deposit protein reserves in the adult or for the generation of new tissue in the young. Further support for this postulate is to be found in the changes in serum and plasma protein, albumin, globulin, fibrinogen, non-protein nitrogen, and amino acid nitrogen levels reported below.

Summer and fall determinations of the total proteins in serum are summarized by age classes in Table I. There was significant difference ( $P < 0.01$ ) between the summer and fall yearling values, also between the summer values for the yearlings and adults.

The total protein in the plasma of the yearling and adult beavers taken during the summer period is 6.37–7.04 g % and 7.04–9.08 g % respectively. The yearlings again show a significantly ( $P < 0.01$ ) lower average value than the adults. The average value of 8.0 g % for the adults is lower than the value of 8.3 g % for the cow, but higher than that found in the goat, sheep, horse, dog, and man (4).

### (c) Albumin

The summer and autumn values for albumin are summarized by age class in Table II. Both the yearling and adult beavers show a lower value during the summer period than in the fall, while the summer values for the yearlings are lower than for the adults. None of the differences was significant.

The blood albumin range of the beaver was from 3.7–5.3 g %, which is slightly greater than the normal human range of 3.5–4.5 g % (12). The mean of 4.4 g % is also higher than that of the horse, cow, sheep, goat, cat, and dog, which have mean values between 3 and 4 g % (4).

TABLE II  
Some normal blood values for the beaver (*Castor canadensis*)

	Yearling beaver			Adult beaver		
	Mean and standard error	Standard deviation	Range	Mean and standard error	Standard deviation	Range
Albumin, g %						
A. Summer level	4.18 ± 0.05	0.17	3.94–4.49	4.28 ± 0.12	0.36	3.78–4.84
B. Autumn level	4.36 ± 0.08	0.25	3.96–4.74	4.51 ± 0.21	0.55	3.86–5.26
C. Total	4.24 ± 0.51	0.25	3.73–4.73	4.37 ± 0.10	0.44	3.78–5.26
Globulin, g %						
A. Summer level	2.64 ± 0.13*	0.46	1.87–3.60	3.32 ± 0.22*	0.68	2.55–4.79
B. Autumn level	2.93 ± 0.08	0.26	2.56–3.52	3.12 ± 0.31	0.83	2.37–4.41
C. Total	2.76 ± 0.08**	0.39	1.87–3.60	3.19 ± 0.17**	0.73	2.37–4.79
Albumin/globulin ratio						
A. Summer level	1.65 ± 0.09*	0.31	1.13–2.26	1.33 ± 0.10*	0.31	0.82–1.79
B. Autumn level	1.50 ± 0.06	0.19	1.13–1.85	1.54 ± 0.17	0.45	0.91–2.07
C. Total	1.58 ± 0.05	0.26	1.13–1.85	1.46 ± 0.08	0.36	0.82–2.07
Fibrinogen, g %						
A. Total	0.47 ± 0.07*	0.17	0.26–0.79	0.58 ± 0.07*	0.24	0.16–1.03

\*, \*\*indicates  $P < 0.05$ . Probability of significance in the appropriate comparisons.

(d) *Globulin*

The data for the globulin levels in beaver blood for the two age classes are given in Table II. In general, the yearlings have a lower level than the adults. This difference is significant ( $P < 0.05$ ) for the summer determinations. The range of 1.8 to 4.8 g % is quite comparable to the normal range in humans, which is 2.0 to 4.5 g % (12). The mean for the adults was 3.2 g %, which is similar to that of the horse (3.25 g %) (4). The cow and cat have higher values while the dog, sheep, and goat have lower average values.

(e) *Albumin-Globulin Ratio (A/G)*

There was considerable variation in the A/G ratios of the beaver, as indicated by the large standard deviation, and no significant difference was indicated between the two age groups (Table II).

Each species of animal shows its characteristic A/G ratio. It must be remembered that this ratio is influenced markedly by the state of nutrition of the animal as well as, and more particularly, by the state of health of the animal. The normal ratio of the horse is unity, while that of the cow is less than unity. The sheep, goat, dog, and cat have an average ratio within the range of 1.1 to 1.5 (4). The normal range for man is 1.2 to 1.8 (12). The beaver has a slightly higher albumin level than the above species, which results in a higher ratio. The mean adult ratio is 1.5 and that of the yearlings 1.6.

(f) *Fibrinogen*

The values obtained for the yearling and adult beaver for fibrinogen are given in Table II. The difference between the two groups is significant although the variability is appreciable. The value of 0.58 g % for the adults is higher than the normal range of 0.2 to 0.4 g % for humans (12). The horse and sheep also have lower average values (0.34 and 0.36 g % respectively), while the goat and cow are higher with values of 0.6 and 0.7 g % (4). The lower values obtained for the yearlings parallel the results found for the albumin and globulin values.

(g) *Non-protein Nitrogen and Amino Acid Nitrogen*

As indicated in Table III, the summer determinations of non-protein nitrogen for both yearling and adult beaver were significantly lower than the autumn values. The adult beaver (28-55 mg %) is higher than the recorded values for other species, including man (20-45 mg %) (4).

The level of amino acid nitrogen in the blood of the beaver ranged between 7 and 12 mg %, which is higher than the normal ranges for man and several domestic species (4-8 mg %) (4,5). The results of the amino nitrogen determinations (Table III) show again that the adults had a slightly higher level than the younger age group, but the difference was not significant.

(h) *Creatine and Creatinine*

The creatine and creatinine values for the different age classes are recorded in Table III. Both constituents show a significantly lower ( $P < 0.01$ ) value for the yearlings as compared with the adults.

The creatine levels in human blood range between 3 and 5 mg %, which is higher than the range of 2 to 3.5 mg % found in the beaver. Creatinine, the least variable nitrogenous constituent in blood, shows a range between 1 and 2 mg in the beaver as well as in man and most domestic species (4, 12).

TABLE III  
Some normal blood values for the beaver (*Castor canadensis*)

	Yearling beaver			Adult beaver		
	Mean and standard error	Standard deviation	Range	Mean and standard error	Standard deviation	Range
Non-protein nitrogen, mg %						
A. Summer level	35.6 $\pm$ 1.3**	4.6	29.6-44.8	36.9 $\pm$ 1.6**	5.2	28.0-44.0
B. Autumn level	45.3 $\pm$ 2.2**	7.3	30.2-54.9	43.6 $\pm$ 2.3**	6.0	36.1-53.2
C. Total	40.5 $\pm$ 1.5	7.5	29.6-54.9	39.0 $\pm$ 1.5	6.6	28.0-53.2
Amino acid nitrogen, mg %	8.93 $\pm$ 0.34	1.17	7.1-11.2	9.28 $\pm$ 0.38	1.27	7.4-12.0
Creatine, mg %						
A. Summer level	2.36 $\pm$ 0.09*,**	0.31	1.96-3.20	2.97 $\pm$ 0.14	0.43	2.02-3.45
B. Autumn level	2.72 $\pm$ 0.13	0.45	1.95-3.29	2.58 $\pm$ 0.13	0.34	2.00-3.00
C. Total	2.53 $\pm$ 0.09	0.41	1.95-3.29	2.81 $\pm$ 0.11	0.43	2.00-3.45
Creatinine, mg %						
A. Summer level	1.23 $\pm$ 0.05	0.16	1.07-1.67	1.56 $\pm$ 0.06	0.18	1.28-1.92
B. Autumn level	1.25 $\pm$ 0.15	0.48	0.90-1.81	1.47 $\pm$ 0.10	0.27	0.95-1.75
C. Total	1.25 $\pm$ 0.07**	0.36	0.90-1.81	1.53 $\pm$ 0.05**	0.21	0.95-1.92
Cholesterol, mg %	113.9 $\pm$ 3.9*	13.0	100-148	96.9 $\pm$ 5.4*	17.1	75-127
Volatile fatty acids, mg $\times 10^{-2}$ %	19.04 $\pm$ 1.51	4.52	10.6-25.5	18.53 $\pm$ 2.03	6.42	9.9-32.3

\*indicates  $P < 0.05$ .

\*\*indicates  $P < 0.01$ .

#### (i) Cholesterol and Volatile Fatty Acids

A few determinations of cholesterol in beaver blood were carried out. From the results (Table III) the difference between the two groups of beaver is significant ( $P < 0.05$ ). The beaver shows an average level of 106 mg %, which is lower than the average value of 152 and 110 mg % exhibited by man and cow respectively. Lower values have been obtained for the cat, rat, rabbit, and guinea pig, which have respective mean values of 93, 52, 45, and 32 mg % (2). It should be noted that the cholesterol value for beaver blood here presented is almost double that for the other rodents. It may be significant to point out that each experimental beaver autopsied during the present study revealed lesions of the aortic vessel that may be related to the high blood cholesterol level. However, it would be premature to infer pathognomic significance to these findings until further work has been completed.

The values for the yearling and adult beaver for blood volatile fatty acids are summarized in Table III. There was no appreciable difference between the two age groups. The volatile fatty acids determined by the procedure used could include the short chain fatty acids such as formic, acetic, butyric, and propionic acids. These are produced during fat metabolism and by bacterial action in the rumen or caecum of a number of animal species.

Man and dog have a normal value of approximately 0.0003 meq (8). This value is considerably below that of the cow as indicated by the 0.0021 meq value obtained in conjunction with the present study. The beaver also has a relatively high level of 0.0019 meq. This indicates a close similarity between the beaver and the herbivore presumably due to the higher level of microbial activity in the caecum and intestine of the beaver.

The present results, together with those previously reported (6), appear to provide sufficient information on normal animals to permit comparison and interpretation of comparable results obtained from wild-taken samples, or from animals subjected to various nutritional regimens.

### Conclusions

In most of the blood characteristics the beaver is similar to the other rodents. The exceptions are a slightly lower erythrocyte count apparently associated with slightly larger cell size, and what appears to be an abnormally high cholesterol level. The significance of these in the adaptation of the animal to its environment merits further study. It is pertinent to emphasize that our experimental animals were all of the race *Castor canadensis leucodontus* obtained from Vancouver Island, and thus living under less extreme environmental circumstances than some other races of the species.

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## THE NAUPLIAR STAGES OF *BALANUS NUBILIS* DARWIN<sup>1</sup>

H. BARNES<sup>2</sup> AND MARGARET BARNES<sup>2</sup>

### Abstract

The details of morphology of the six naupliar stages of *Balanus nubilis* are described. The nauplii are particularly characterized by their large size and in the later stages by a distinctly pentagonal outline. The general pattern of development conforms to that previously found for balanid nauplii. Tables are given of sizes and setation formulae.

### Introduction

Descriptions of cirripede larvae, sometimes referable to known species, have been available for many years (6). Nevertheless, only comparatively recently have the complete series of naupliar and cyprid stages of some common species become known (see ref. 4 for a list); many more remain to be described. Apart from the intrinsic value to our understanding of the morphology and systematics of the Cirripedia, recognition of the free-living larvae is an essential prerequisite for studies on the planktonic ecology of any species. *Balanus nubilis* was first described by Darwin (5) (see also ref. 12) and a supplementary account has been given by Henry (7), who considers that *B. flos* Pilsbry (11, 12) and *B. altissimus* Cornwall (3) are synonymous with *B. nubilis*. According to Henry (7) the species occurs along the Pacific coast of America from southern Alaska to San Quentin, Baja California; towards the south it is common in the lower intertidal of exposed coasts (14); in the Puget Sound region it is more frequent in restricted parts of the sublittoral, where it reaches a large size (up to 10 cm basal diameter) and where enormous aggregates with individuals crowded upon each other are found.

### The Material and Methods

The second and third naupliar stages were raised in laboratory culture from stage I nauplii that had been hatched from ripe egg masses carefully removed from the mantle cavity of an adult. The remaining naupliar stages (IV-VI) were not successfully cultured but, since the earlier stages had been raised in culture, could be easily recognized in plankton collections; during the summer months the larvae of *B. nubilis* form a considerable proportion of the cirripede larvae present in plankton collections taken in waters adjacent to Friday Harbor, San Juan Archipelago, Washington. The cypris stage was not determined. The plankton samples were preserved in formalin and

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<sup>2</sup>The Marine Station, Millport, and The University of Washington Laboratories, Friday Harbor.

the stages separated. The measurements and drawings (except for stage I which only rarely is taken in plankton tows) were all made from the planktonic material rather than from the laboratory cultures; description of any abnormalities arising in the latter is thereby avoided. After separation of the stages, individuals were dissected with fine needles under a binocular microscope and the appendages drawn directly or after first mounting in a methylene blue - polyvinyl alcohol medium in which the spines are more easily seen.

## Results

### *General Features of the Nauplii*

Six naupliar stages were found; outline drawings are given in Fig. 1 and various dimensions (based on 20 individuals) are set out in Table I. The total length was measured from the tip of the caudal spine to the frontal margin, the carapace length from the frontal margin to the posterior border of the carapace, and the carapace width was taken as the greatest width of the body behind the frontolateral horns.

TABLE I  
Sizes ( $\mu$ ) of larval stages of *Balanus nubilis*

Stage	Total length	Carapace			Frontolateral horns
		Width	Length	Spines	
I	266	157	—	—	18
II	433	201	—	—	60
III	469	252	—	—	81
IV	616	333	410	69	89
V	790	480	541	86	114
VI	1065	663	744	162	189

Compared with most other species whose larvae have been described, those of *B. nubilis* are large and bulky, being comparable in this respect with *B. balanoides*; the contrast in the size of the adults is not evident in the larval stages. The larvae are characterized by the following features which are most obvious in the later stages. The frontal margin of the carapace (except in stages I and II) is relatively straight as are the lateral margins; posteriorly, the latter curve sharply towards the mid-line, so that the general outline of the nauplius is pentagonal; in this respect they resemble somewhat the much smaller larvae of *B. perforatus* (9). In stages IV-VI the frontolateral horns and carapace spines are prominent. The abdominal process is only a little shorter than the caudal spine.

In stage I (Fig. 1) the body is more or less pear-shaped, the distinct pentagonal appearance being not yet evident. As is usual in freshly liberated balanid stage I nauplii the frontolateral horns are directed posteriorly and more or less appressed to the sides of the body. The caudal process is relatively short and there are no frontal filaments. In this, as in all stages, a median eye is present.

In stage II the frontolateral horns are prominent and project almost at right angles to the long axis of the body; the pentagonal outline is now more evident although the frontal margin of the carapace is still somewhat bowed. Frontal filaments are present in this and all subsequent stages. The abdominal process is almost equal in length to the caudal spine. The former bears a pair of spines and forms at its posterior extremity a well-defined furca whose diverging rami are slender and straight.

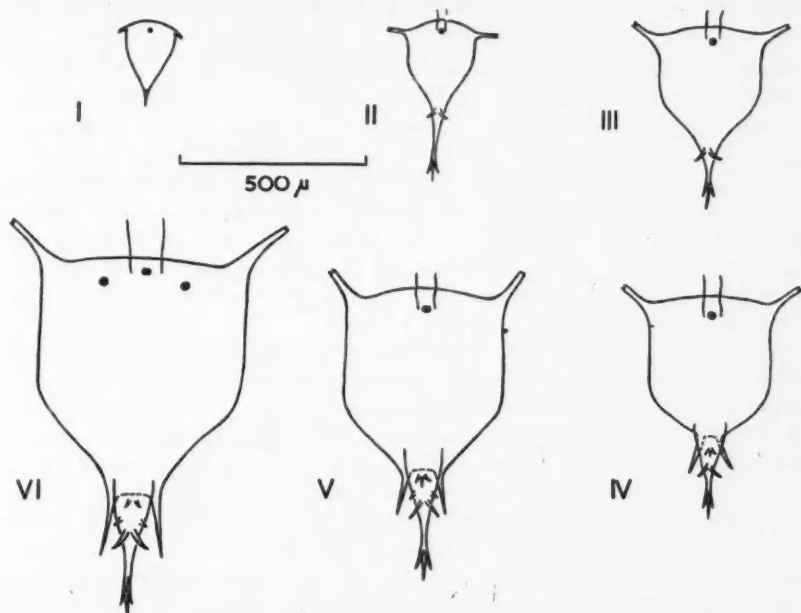


FIG. 1. Outline drawings of the six naupliar stages of *Balanus nubilus* showing shape and the more important spinous processes.

In stage III the frontolateral horns project more to the anterior; the frontal margin of the carapace is less bowed and the pentagonal outline is now quite distinct. The abdominal process still bears only one pair of spines. The antennule has a single preaxial seta not present in stage II.

In stage IV the posterior border of the carapace is clearly delimited and bears a pair of long, straight but tapering carapace spines set rather close together when compared with their relative position in some other species.

Stage V, apart from being larger, closely resembles stage IV in general appearance. A further pair of abdominal spines are present.

Stage VI, a bulky nauplius, has the abdominal process considerably enlarged and the cirriform appendages, now fully segmented, may be clearly seen underneath the exoskeleton. In addition to the median eye found in all the earlier stages, paired lateral eyes are now present; however, in the specimens examined the paired eyes were rarely deeply pigmented.

### *The Carapace*

A distinct posterior border to the carapace, with a pair of spines arising from it, is first evident in stage IV. This is in agreement with the statement of Jones and Crisp (8) that only at this stage do these features develop in balanid nauplii; as these authors point out, earlier accounts of a carapace border with spines in stage III of *B. balanoides* and *B. crenatus* (1, 13) are probably erroneous. In *B. nubilis* the carapace spines are long, reaching a size of 160  $\mu$  in stage VI, and are set quite close together: they are straight and taper towards their extremities.

### *Abdominal Process and Caudal Spine*

The abdominal process, first clearly marked out in stage II and present in all subsequent stages, is similar to that of other balanid nauplii; arising in a mid-ventral position just posterior to the pendant lip of the labrum it projects ventrally and posteriorly. It is separated from the caudal process by a deep notch. The abdominal process has a swollen proximal part in which are the developing thoracic appendages of the adult, and a terminal portion consisting of stem and furca. Laterally are two raised areas bearing long fine hairs; these, present in other species, represent the position in which the maxillules develop.

A single pair of abdominal spines is present in stages II and III. In stage IV a further pair of smaller spines, arising more to the anterior and with a single median spine between them, is present. Stage V has yet a further pair of stout spines anterior to the first series and arising in a more dorsolateral position on the process. The median spine of the second series is lost in stage VI which has, therefore, only six spines. The arrangement of these spines is identical with that described by Norris and Crisp (9, Table 2, p. 399) for *B. perforatus* and by Jones and Crisp (8) for *B. improvisus*. The similar arrangement in *B. nubilis* adds strength to the view that the development of the full series of spines just described is not, as was suggested by Buchholz (2), associated with abnormal growth. In stage VI there is a series of paired, anteriorly projecting spines containing the tips of the cirri developing within the abdominal process. Differentiation of the associated muscle blocks is very evident in stage VI nauplii. The furca of the abdominal process extends almost to the tip of the caudal spine; the two rami of the former are well developed and diverge at an angle of about 45°. In the later stages both the abdominal process and the caudal spine are somewhat shorter relative to the carapace length.

### *The Labrum*

This is trilobed as in other *Balanus* sp., with the larger median lobe projecting somewhat beyond the smaller lateral lobes; the lobes are all setose.

### *The Eyes*

A median naupliar eye is present in all stages. In stage VI there is in addition a pair of laterally placed compound eyes. The latter were rarely deeply pigmented in the individuals examined. Jones and Crisp (8) have drawn attention to variability in the pigmentation of these paired eyes;

they state that in *B. improvisus* compound but unpigmented eyes are readily seen in stage V nauplii and two clear areas, which foreshadow their development, may be readily made out in even the earlier naupliar stages.

### Setation of the Appendages

Paired antennules, antennae, and mandibles are present in all stages. Stages II–VI have frontal filaments (stylets) that arise on either side of the median eye and stages IV–VI have maxillules, lying at the side of the abdominal process.

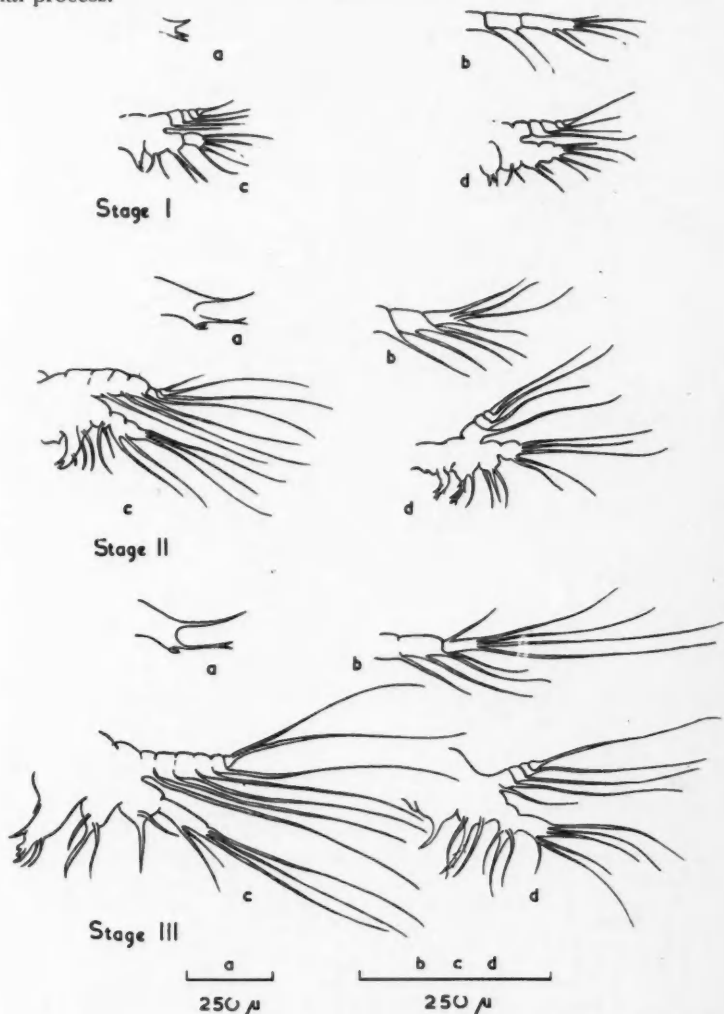


FIG. 2. Stages I, II, and III larvae of *Balanus nubilis*: (a) lateral view of abdominal and caudal processes, (b) antennule, (c) antenna, (d) mandible.

Figures 2-4 show the setation of the appendages and their form, adopting Bassindale's (1) notation, which is now conventional, is set out in Table II. The antennule resembles that of other species; a preaxial seta is first found in stage III and a further one added in stage IV. Postaxial setae are successively added in stages V and VI. The sucker of the cyprid can be seen within the swollen third segment in stage VI. The setation of the antenna of the first two stages is similar to that of most other species and, as is commonly found, there is a noticeable addition of setae at stage IV. Later

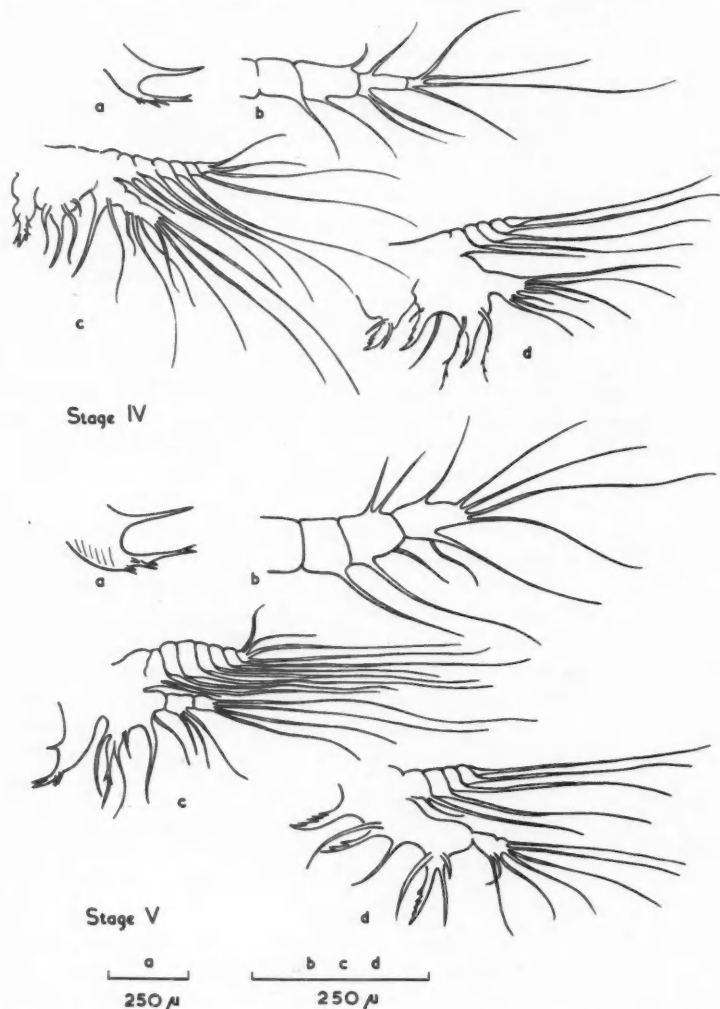


FIG. 3. Stages IV and V larvae of *Balanus nubilis*: (a), (b), (c), and (d) as in Fig. 2.

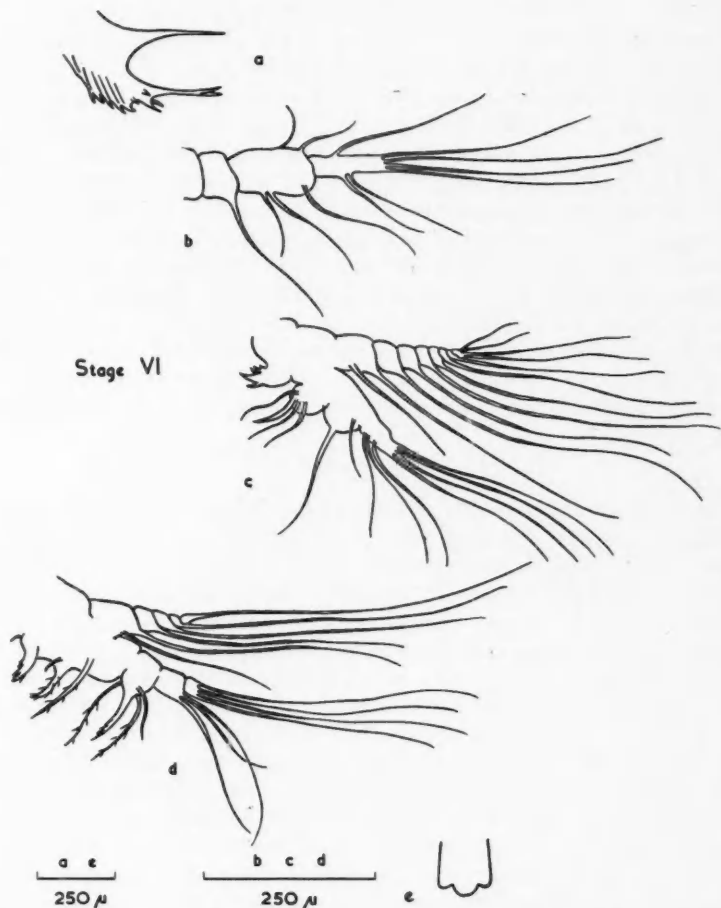


FIG. 4. Stage VI larva of *Balanus nubilis*: (a), (b), (c), and (d) as in Fig. 2; (e) labrum.

TABLE II

Setation formulae for the naupliar stages of *Balanus nubilis* (all notations according to that given by Bassindale (1))

Stage	Antennule	Antenna	Mandible
I	0.4.2.1.1	0.1.4.-0.3.2.2.2.G	0.1.3.-0.3.2.2.2.G
II	0.4.2.1.1	0.2.5.-0.3.2.2.2.G	0.1.3.-0.3.2.3.2.G
III	1.4.2.1.1	0.2.5.-0.3.2.2.3.G	0.1.4.-0.3.3.3.3.G
IV	1.1.4.2.1.1	0.3.6.-0.4.3.2.4.G	0.1.4.-0.4.3.3.3.G
V	1.1.1.4.2.1.1.1	0.4.7.-0.4.3.2.4.G	0.1.5.-0.4.3.4.3.G
VI	1.1.1.4.2.1.2.1	0.4.8.-0.4.3.2.4.G	0.1.5.-0.4.3.4.3.G

stages resemble in their setation some other species described but any resemblances or differences appear to have no specific significance. The gnathobase is as usual covered with a complex series of setae and terminates in a bifid prong-like process. The mandibular setation varies little from one species to another: its gnathobase possesses a simple pointed process bearing stiff setules. A comparison of the setation formula so far available indicates that they are of little diagnostic value for the separation of species or even genera (*B. improvisus* and *Elminius modestus* are, for example, identical).

Variability in the setation of naupliar appendages has been commented upon by Norris, Lovegrove, Jones, and Crisp (10; see also ref. 8). The suggestion was made that variability is particularly associated with cold-water species having a relatively longer period of development. Very little variation was found in the setation of *Balanus nubilis* in collections made over several days during the summer months. The absence of variation in this and in *B. eburneus* (4), also a warm temperate species, supports this suggestion.

### Discussion

Once the shape of the nauplii of *B. nubilis* is recognized the separation of the individual stages can be largely made on size: the relatively small variation of length within a stage indicates that in general plankton work, any errors introduced by using size would be negligible. Other characters may, however, be used. Stage I is rarely present in planktonic collections, but may be recognized by the posteriorly directed frontolateral horns. The stages from II-VI may be separated by the setation of the antennule, which is relatively easily examined. The large size, presence of a preaxial seta on the antennule, and the more distinct pentagonal outline are useful for the separation of stage III from stage II without any further dissection. Stage IV is readily separated from stage III by the clearly delimited posterior border of the carapace with its two spines. Stage V, apart from its larger size, closely resembles stage IV. It may be distinguished by the additional pair of abdominal spines, which are quite readily seen. Stage VI is altogether more bulky, three-eyed when fully developed (although the paired eyes need not be deeply pigmented), and development of the cirriform appendages is well advanced.

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## MEASUREMENT OF SOME PHYSIOLOGICAL PARAMETERS IN RAINBOW TROUT (*SALMO GAIRDNERII*)<sup>1</sup>

R. H. SCHIFFMAN<sup>2</sup> AND P. O. FROMM

### Abstract

Data on physiological parameters such as hematocrit, hemoglobin, erythrocyte count and size, organ weights, body-water weight, plasma and blood volumes are given for hatchery-raised *Salmo gairdnerii*. Methods for obtaining the above data using fingerlings varying in weight from 9 to 29 g are discussed. Mean values reported are as follows: hematocrit 31.8; hemoglobin 6.51 g/100 ml blood; R.B.C. count 1.11 million/mm<sup>3</sup>; R.B.C. length 14.67  $\mu$ . Kidney, liver, and spleen weights as well as total body water, and plasma and blood volumes were found to be statistically valid factors of body weight. Straight-line regression equations are given for each. A comparison of these data with recent summaries of similar data is given and possible reasons for discrepancies are discussed.

Recent summaries of the literature dealing with the physiology of fishes (Prosser *et al.* (7), Spector (8), Brown (1)) have shown a notable lack of information concerning many species. More reports are needed giving results of many experiments so that statistical evaluation of data is feasible. Rainbow trout are raised in many hatcheries for stocking purposes and are consequently available for use as an experimental animal during most of the year. The information reported herein is aimed at the further development of the hatchery-raised rainbow trout for physiological research and, in our own case, to define physiological parameters for possible use in toxicity experiments. This report deals primarily with blood physiology and the relation of body weight to total body water and to organ weights.

To obtain physiological data from small fish extensive modifications of methods used in experiments with larger animals are required. The extent of these modifications and the reliability of the various procedures are given below.

### Materials and Methods

All fish used in these experiments were obtained from the Michigan Department of Conservation, Wolf Lake hatchery. At the hatchery these fish were kept in ponds and they fed upon the natural food available. They were brought to the laboratory in lots of 100 and were kept in aquaria (26 gal capacity) in a constant temperature room under continuous illumination.

The water had the following characteristics: temperature, 14°–15° C; hardness, 334 mg CaCO<sub>3</sub>/l.; alkalinity, 204 mg CaCO<sub>3</sub>/l.; and pH, 8.5 to 8.8.

<sup>1</sup>Manuscript received August 20, 1958.

Contribution from the Department of Physiology and Pharmacology, Michigan State University, East Lansing, Michigan, U.S.A.; supported in part by grant No. RG 4009 from the National Institutes of Health; and published with the approval of the director, Michigan Agricultural Experiment Station, as journal Series No. 2241.

<sup>2</sup>This paper is based on a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate College of Michigan State University. Present address: Biology Operation, Hanford Laboratories, General Electric Company, Richland, Washington, U.S.A.

Each Monday, Wednesday, and Friday the fish were fed a balanced diet of dried pellets currently used by the Michigan Department of Conservation. The formula for these pellets is unknown to the authors, since this information would not be released by the Department.

The fish were fingerlings with a total length ranging from 11.3 to 15.7 cm and wet weights from 8.7 to 29.1 g. This small size was used since these fish are easily handled and they conform to the specifications recommended by Doudoroff *et al.* (2) in their toxicity bio-assay procedure.

With the aid of a dissecting microscope, organs were removed from fish and weighed on a Roller-Smith balance. The remainder of the carcass, after it was weighed, was dried in an oven at 95° to 105° C for 48 hours. Owing to previous study, samples of this size were considered to be at constant weight within this time. The loss in weight resulting from drying was considered a measure of the total body water. The error caused by removal of the organs will be discussed.

Fish were anesthetized by immersion in a solution containing 300 mg tricaine methanesulphonate (MS-222)/l.

After the fish were anesthetized they were placed on their dorsum in a V-shaped trough, and a mid-ventral incision made to expose the heart. Blood, obtained by puncture of the ventricle, was collected in a 0.25-ml syringe which had been rinsed with 1000 USP units/ml heparin solution and fitted with a 27-gauge needle. No blood sample of less than 0.1 ml was used since it was felt that in smaller samples the residue of heparin would cause a significant dilution error. The average blood sample was between 0.1 and 0.2 ml. Most of the blood thus collected was expressed onto a wax surface, with care being taken to avoid the formation of bubbles. The small remainder, that most likely diluted with heparin, was used to make a thin smear which was air-dried and stained with Wright's stain.

From the puddled sample a van Allen hematocrit tube was filled to the 100 mark and then 0.6% saline drawn into the tube until the bulb of the tube was about one-half full. The tubes were centrifuged at 2500 r.p.m. for 20 minutes and read. To test the reproducibility of this determination, four analyses were made from a single blood sample. The resultant standard error was 0.72 ml/100 ml.

The hemoglobin content of fish blood was determined colorimetrically by the acid-hematin method as outlined in the Bausch and Lomb Spectronic 20 Clinical Technique Manual. Nuclei in the hemolyzed samples were removed by centrifugation at 2500 r.p.m. for 10 minutes. Transmission of light through the supernatant solution at a wave length of 525 m $\mu$  was read using the colorimeter. The standards for human blood, provided in the manual, were used and results are reported in grams of hemoglobin per 100 g of blood. Results of six determinations of hemoglobin in a single blood sample had a standard error of 0.32 g/100 g.

A standard red blood cell pipette was filled from the puddled blood and a 1/200 dilution was made, again using 0.6% NaCl as the diluent. The erythrocyte count was then made in the accepted clinical manner using a Levy hemocytometer.

Red cell lengths were determined from the air-dried smears. Random fields were brought into focus and all cells that would align properly as the ocular micrometer was revolved were measured.

Measurement of the plasma volume was done by the dye dilution method using Eastman's T-1824 (Evans blue). The heart was exposed, as noted above, and the dye was injected from a 1.0 ml tuberculin syringe fitted with a 30-gauge needle. The syringe was manipulated using a microtitration apparatus so that exactly 0.012 ml containing 0.074 mg of the dye was injected per animal. If bleeding as a result of the incision or ventricular puncture was profuse the fish was discarded. To counteract the error from a slight amount of bleeding, the volume of the dye was not subtracted from the final result. The fish were kept on their dorsum in a wet, gauze-lined, V-shaped trough, and artificial respiration was induced by means of water flowing over the gills.

A period of 10 minutes was allowed for distribution of the dye throughout the circulatory system, after which a blood sample was obtained by heart puncture. An aliquot was removed for a hematocrit determination and the remainder was centrifuged in a 1-ml centrifuge tube at 2500 r.p.m. for 10 minutes. Exactly 40 cu. mm of supernatant plasma was diluted with 0.5 ml of distilled water in a cuvette. The transmission of light was measured using a Bausch and Lomb Spectronic 20 (650 m $\mu$ ), and the concentration of dye in the plasma determined from a standard curve. From plasma volume and hematocrit data the circulating blood volume was calculated.

All data showing definite relationship to total wet-body weights were analyzed as a regression of these values on body weight rather than the artificial approach: per cent of the body weight. Not only are the weights of tissues or volume of fluids a factor of body weight but also the per cents of body weights show a regression. Heroux and Gridgeman (3) present excellent arguments for this type of reasoning.

## Results

### *Organ Weights*

All regression and correlation coefficients proved to be highly significant and the calculated regression curves are illustrated in Fig. 1. The equations for these lines, including the standard error of the estimate, are as follows:

$$\text{Kidney: } Y \pm 20.43 \text{ mg} = -10.50 + 8.57X \text{ g.}$$

$$\text{Liver: } Y \pm 40.46 \text{ mg} = -38.95 + 12.89X \text{ g.}$$

$$\text{Spleen: } Y \pm 11.53 \text{ mg} = -7.93 + 2.97X \text{ g.}$$

"Y" is the predicted weight of the organ in milligrams at "X" grams of body weight.

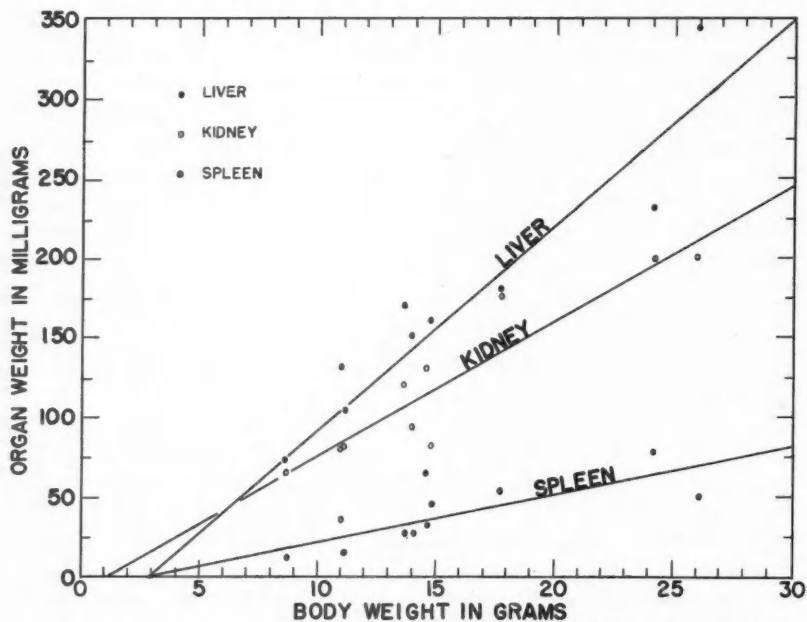


FIG. 1. Graph showing relation between total body weight and organ weight of kidney, liver, and spleen.

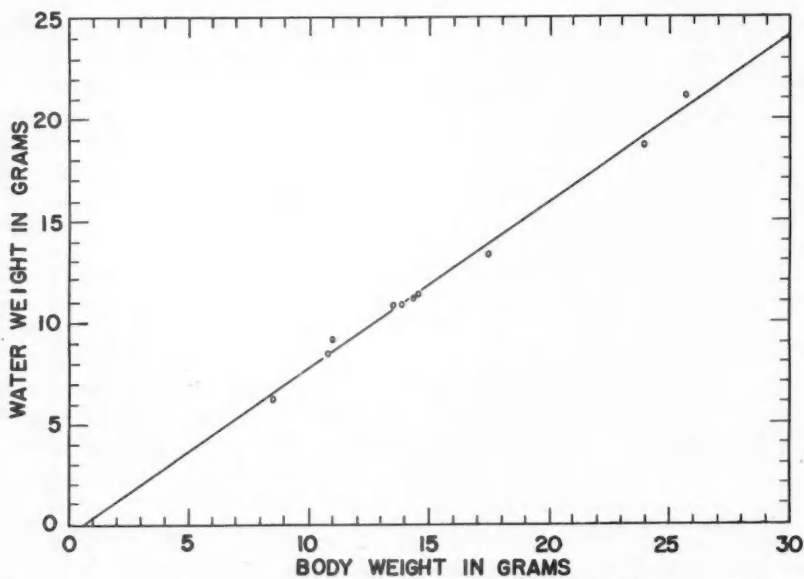


FIG. 2. Graph showing relation between total body weight and body-water weight.

### Total Body Water

These data, from 10 fish, were analyzed in the same manner as the organ weights. Once again the regression and correlation coefficients proved to be highly significant and the following regression equation was calculated (see Fig. 2).

$$Y \pm 0.47 \text{ g} = -0.62 + 0.82X \text{ g.}$$

The main objection to the method used is that certain organs were removed prior to the determination of total body water; however, the error introduced by this procedure is small. Assuming the organs to be 90 to 100% water, an extreme estimate, the maximal error introduced by removal of these organs would be 1.4%.

### Hematocrits

The mean hematocrit was found to be  $31.8 \pm$  a standard error of 1.39 ml of red blood cells per 100 ml of blood. Information compiled by Spector (8) indicates an average hematocrit for rainbow trout of 27.2 ml R.B.C./100 ml of blood, with a range of 22 to 36.

### Hemoglobin

The mean of the hemoglobin determinations on 10 fish was  $6.51 \pm$  the standard error of 1.27 g/100 g of blood. Spector (8) reports a hemoglobin value for trout of 8.5 g/100 ml, with a range of 6.2 to 11.5. Prosser *et al.* (7) give a range from 8.7 to 17.6 g/100 ml for several species of fish.

TABLE I

Summary of values of physiological parameters investigated  
(All means were calculated from samples of 10 fish. \*Calculated from regression equations using  $X = 100 \text{ g}$ )

Determination	Mean	Standard error
Hematocrit (% R.B.C.)	31.8	1.39%
Hemoglobin (g/100 ml blood)	6.51	0.40 g
R.B.C. count (millions/cu. mm)	1.11	0.03 million
R.B.C. length ( $\mu$ )	14.67	0.09 $\mu$
Kidney weight as % of total body weight	0.85%*	
Liver weight as % of total body weight	1.25%*	
Spleen weight as % of total body weight	0.29%*	
Body water as % of total body weight	81.0%*	
Plasma volume as % of total body weight	1.50%*	
Blood volume as % of total body weight	2.25%*	

The usual methods for the determination of hemoglobin call for a colorimetric analysis of hemolyzed blood at some point during the procedure. If the interfering nuclei are not removed prior to this manipulation, the results will be high. The authors made a few determinations of hemoglobin prior to removal of the nuclei and found these values agreed quite well with the upper ranges published by Spector. This factor probably accounts for the fact that the data reported herein fall in the lower part of the range of data reported in the literature.

### Erythrocyte Count

The red blood cell counts on 10 fish average  $1.11 \pm 0.03$  million cells/cu. mm. This agrees very well with Spector's value for trout of 1.01 million cells/cu. mm, with a range of 0.74 to 1.5 million cells/cu. mm. Prosser *et al.* (7) report a range for fish of 0.585 to 2.685 million cells/cu. mm.

### Red Blood Cell Length

By taking the average length of 10 cells/fish and using 10 fish a value of  $14.67 \mu \pm$  the standard error of  $0.092 \mu$  was determined. This value agrees well with the average of  $16.7 \mu$  reported for trout by Prosser *et al.* (7).

### Plasma Volume and Blood Volume

The regression and correlation coefficients, relating plasma and blood volume to total body weight, were found to be highly significant. The following prediction equations, including the standard error of the estimate, were calculated and the regression curves plotted in Fig. 3.

$$\text{Plasma volume: } Y \pm 0.07 \text{ ml} = 0.094 + 0.015X \text{ g.}$$

$$\text{Blood volume: } Y \pm 0.08 \text{ ml} = 0.15 + 0.023X \text{ g.}$$

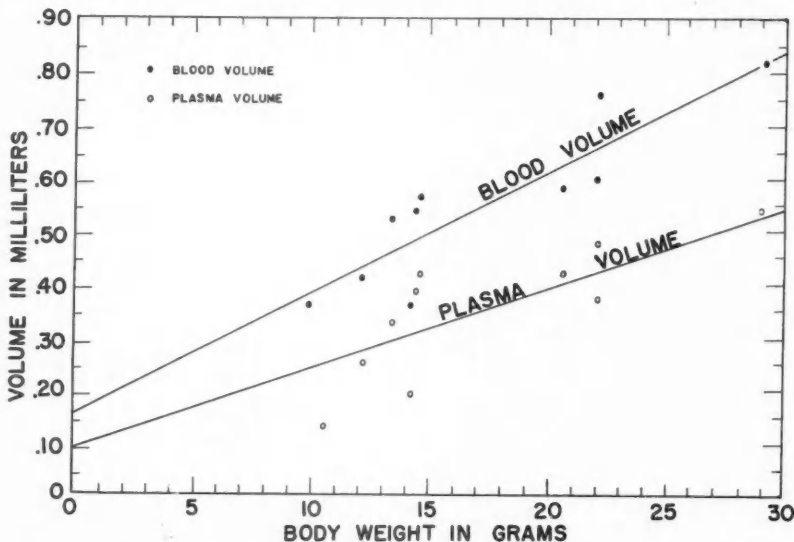


FIG. 3. Graph showing relation between total body weight, and plasma and blood volumes.

### Discussion of Regression Data

We must realize extrapolation of regression lines beyond the range of experimental data may be invalid. Although this may be the case, extrapolation and subsequent conversion of the calculated weight to fraction of total body weight yields a relatively stable percentage above 100 g. Assuming

these values calculated for a hypothetical 100-g fish to be an indication of values found in an adult fish we can then compare them with values presented in the literature.

No information on the relation of organ weight to body weight for hatchery-raised rainbow trout or members of the family Salmonidae was found in the literature. Krumholz (4) reported organ-weight data for eight species of warm-water fish which had been exposed to low level radioactive wastes. The 100-g calculated percentage of our data are 0.8%, 1.25%, and 0.29% for the kidney, liver, and spleen respectively. Our data for kidney and liver compare favorably with Krumholz' results of 0.11% to 0.98% for kidney and 0.84% to 3.65% for the liver. The data on spleen weight reported by Krumholz ranged from 0.06% to 0.14%. Species differences are definitely indicated by Krumholz' data on warm-water fish and this factor may well account for the deviation, from his results, of our data on rainbow trout.

Love (5) states that the results of total body-water determinations are difficult to compare owing to the widely differing techniques used by various authors. He concluded that fish in general contain about 80% to 85% water, with extreme values ranging from 53% to 89.3%. Our data for rainbow trout, calculated for a 100-g fish to be 81%, fall well within the range for other species.

Prosser *et al.* (7) reported a blood volume for teleost fishes of 1.5% to 3% of body weight, and Mott (6) summarized data on Actinopterygii and reported a range of 1.4% to 2.8% of body weight. The regression equations show that the amount of trout plasma and blood decreases in volume in relation to body weight as the animal increases in size, a characteristic of most animals. Our hypothetical 100-g trout would have a calculated plasma and blood volume of 1.50% and 2.25% respectively. These values agree well with the reported data.

### Summary

1. Methods for the collection of hematocrit, hemoglobin, erythrocyte count and size, organ weights, body-water weights, and plasma and blood volume data are given.
2. Kidney, liver, and spleen weights; total body water; and plasma and blood volumes were found to be statistically valid factors of body weight. Straight-line regression equations are given for each.
3. For convenience Table I summarizes the results of all data obtained.

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## METHODS FOR STUDYING FORCIPOMYIA MIDGES, WITH SPECIAL REFERENCE TO CACAO-POLLINATING SPECIES (DIPTERA, CERATOPOGONIDAE)<sup>1</sup>

L. G. SAUNDERS

### Abstract

The known pollinators of cacao are shown to belong to the subgenera *Proforcipomyia* and *Thyridomyia* of the genus *Forcipomyia*. Characters are presented for the recognition of larvae, pupae, and adults of members of these groups. The breeding grounds are reported for four potential pollinating species, which are described and figured in all stages. Methods of collecting, rearing, and studying these insects are given, including rearing on artificial cultures.

### Introduction

Thanks to the work of Billes in Trinidad (2), Posnette in Trinidad (5) and West Africa (6), and Soetardi in Java (8), it can be accepted as proved that the pollination of cacao (*Theobroma cacao* L.) is brought about largely, if not exclusively, by female midges of the genus *Forcipomyia*. Even when so scarce as to be extremely hard to find, the female midges still occasion enough "set" to build up a satisfactory crop over a period of flowering; but pollination ceases if their numbers drop to virtual absence, for it has never been shown that mechanical transfer of pollen, or any other insects associated with cacao flowers, can take their place. A knowledge of their biology is, therefore, of fundamental importance to explain their habits and seasonal fluctuations, and suggest measures which might be adopted to provide better breeding grounds or otherwise increase their numbers.

At St. Augustine, Trinidad, B.W.I., in the summer of 1957, I was able to collect and rear the early stages of three potential pollinating species, even though the population was at a very low ebb. These three, and one taken in Costa Rica, are described, together with the females of two species known to be pollinators. Methods and techniques are added as a guide for future workers in the field.

### Classification of Species Responsible for Pollination

Of the eight established subgenera of *Forcipomyia* (7) two contain all the species so far known to pollinate cacao: *Proforcipomyia* has most of the species and individuals taken in cacao flowers; *Thyridomyia* has few. All Posnette's collections were identified by Macfie (4), whose descriptions and figures make it possible to assign the species to the subgenera mentioned. Moreover, Carter's (3) description of the larvae and pupae of *F. ingrani*, an important African species, shows that it unquestionably belongs in the subgenus *Proforcipomyia*.

<sup>1</sup>Manuscript received October 20, 1958.

Contribution from the Department of Biology, University of Saskatchewan, Saskatoon, Sask.

It is most unfortunate that the only stage visiting cacao flowers, the female, is the most difficult to identify. As an example, Macfie, an experienced taxonomist, tacitly admitted that his *F. quasi-ingrami* (4) was a multiple species, because he received only females and was unable to sort them out. The males are of more use taxonomically on account of their genitalia, but they can rarely be associated with females of the same species except by rearing. The larvae and pupae have the best characters, and there can be no mistake of specific identity once the early stages are described and figured. Consequently, the larva is now nearly always designated holotype in descriptions of *Forcipomyia* and *Atrichopogon* species, when it is available.

#### GENUS *Forcipomyia*

These are ceratopogonid midges of small to very small size, with wing length 0.6 mm to 1.4 mm, measured from arculus to wing tip (wing length is constant within narrow limits, whereas body length may vary greatly under distention with food or eggs). The presence of empodia between the claws characterizes all members of the Forcipomyiinae and therefore holds for *Forcipomyia* with the exception of males of the subgenus *Trichohelea*, and not all of those lack them. The wings are thinly or densely clothed with macrotrichia, and sometimes patterned; the first radial cell is always short and practically closed, the second may be closed or open and is short, bringing the end of the costa not much beyond the middle of the wing. By contrast, a practically bare wing with elongate second radial cell is the main distinguishing character of the closely related genus *Atrichopogon*. The tarsal ratio (T.R., the ratio of the second to the first tarsal segment of the third leg) varies from 0.5 to 3.2; it is of some use in distinguishing subgenera and more helpful in specific identification. The male genitalia are fairly constant in the form of the basistyles, dististyles, and shield-shaped aedeagus; on the other hand the variation in the parameres and internal apodemes is of great use in distinguishing the different subgenera.

The larvae are elongate, distinctly segmented, bearing many different kinds of setae on the body and the hypognathous head; they have eversible prothoracic and anal pseudopods, whose terminal hooklets may be useful. However, they are so variable throughout the subgenera that it is impossible to summarize them all here, and figures and descriptions will be provided of the important ones. The pupae are less variable and not readily confused with those of other genera except perhaps *Atrichopogon*, but again a figure can tell more than many words.

#### SUBGENUS *Proforcipomyia*

Females are dull brownish grey with unmarked wings densely haired with slender macrotrichia. If a specimen has this coloration and the tarsal ratio is between 1.3 and 2.0, it is safe to conclude that it belongs here and not in the more common subgenus *Forcipomyia*, although an overlapping of tarsal ratios can be demonstrated. The fourth and fifth palpal segments vary between

distinct separation and complete fusion, usually being in a state of incomplete fusion. There may be sudden elongation of antennal segment 11 compared with 10, but more often the transition to the last five somewhat longer segments is gradual (an insignificant first segment, the scape, before the globular second, the pedicel, is never figured). In the wing the first radial cell is obliterated, or at the most represented by a short median groove; the second is open and short. The tibial comb of the foreleg furnishes useful characters to separate species. There are two spermathecae, equal or unequal in size.

Males match the females in dull, dark coloration, and their tarsal ratio and wing venation are similar. The genitalia supply the most distinctive character for adults of the subgenus: the internal basistylar apodemes join to form an arch from which no evident parameres proceed backward (rudimentary lobe-like parameres are demonstrable in some species by careful preparation and staining). Other features of the genitalia usually follow a constant pattern of short, broad basistyles, broad dististyles hollowed on their anterior face, and simple triangular aedeagus. The species *mortuifolii* described below is at present unique in showing striking variation in the form of the dististyles and the ninth sternite.

Larvae show good subgeneric characters. There is always some pigmentation in the dorsal subcutaneous fat body resulting in a pinkish-brown pattern on the thorax or throughout the length of the body, faint, moderate, or strong in intensity. Morphologically the most distinctive character is the pointed elongation and fringing of the cauda at the tip of the body. The antennae are usually slender and backward-curving, and the profile of the head has two flattened or concave areas in place of the smoothly rounded profile of larvae in all other subgenera. The prothoracic pseudopod is a simple spinulose cushion; it bears slender apical hooklets in only two known species, one of which is described in this paper.

Pupae have one character found in no other subgenus: the prothoracic horns bear a partial or complete circlet of spiracular papillae arranged in upper and lower palisades. The genital processes of the male pupa are dorsal, and the larval skin is rarely retained on the tail.

*Forcipomyia (Proforcipomyia) mortuifolii* Saunders, new species

*Larva*.—Length 2.3 mm. Color: reddish-brown pigment pattern throughout body, fading slightly posteriorly. Head dark, profile distinctly scalloped; *p* hairs fine, spear-shaped, *q* hairs half-spear, stouter (Fig. 1, K). *a* hairs of body spear-shaped with only slightly swollen tip; short, stout, wedge-shaped on prothorax (Fig. 1, I); dorsolateral hairs short, stout, curved; four short, fine lateral hairs on each segment. Prothoracic pseudopod the normal spinulose cushion. Cauda short, reaching as far as the tips of the last, fused-based *a* hairs which are unusually short (Fig. 1, J).

*Pupa*.—Length 1.8 mm. Color (exuviae) pale yellowish throughout. Three tubercles on median sclerite of head, none on laterals. Thorax with two pairs conspicuous pointed tubercles and one posterior pair smaller, rounded. Abdominal segments with only minute lateral setae; terminal

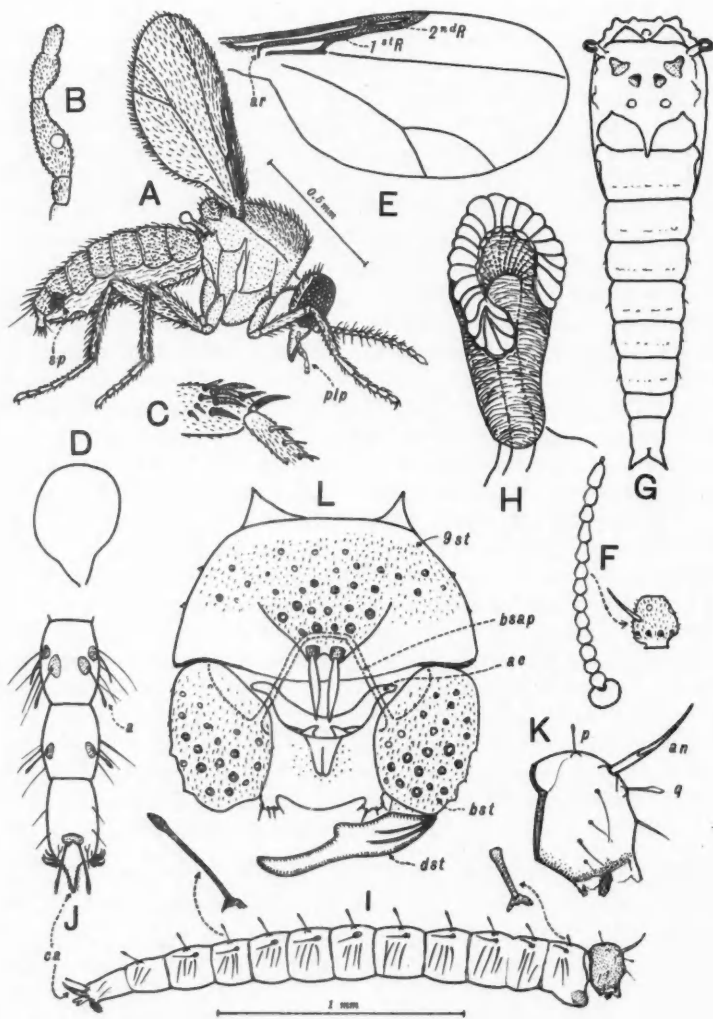


FIG. 1. *Forcipomyia* (*Proforcipomyia*) *mortuifolii*. A, entire female; B, female palp; C, foretibial comb of female; D, outline of spermatheca; E, female wing venation; F, female antenna; G, pupa; H, pupal prothoracic horn; I, entire larva; J, last three segments of larva; K, larval head; L, male genitalia. *ae*, aedeagus; *an*, antenna; *ar*, arculus; *bsap*, basistylar apodeme; *bst*, basistyle; *ca*, cauda; *dst*, dististyle; *plp*, palp; *sp*, spermatheca; *9 st*, ninth sternite.

processes divergent in both sexes (Fig. 1, G). Prothoracic horn without basal enlargement, bearing incomplete, elongate circlet of spiracular papillae, about 25 in lower palisade (Fig. 1, H).

*Female*.—T.R. 1.5. Wing  $0.6 \times 0.3$  mm. Ground color reddish brown as in larva; thorax and abdomen greyish brown, only slightly darker than pleura and sternum; legs pale yellowish; halteres pale, not chalky white. Antennae with basal segments globular, gradually becoming elongate to 11, 12 and 13 abruptly longer, 14 shorter again; one slender hyaline sensory hair per segment, shorter than length of segment (Fig. 1, F). Palps with delicate circular sensorium on third segment, fourth and fifth segments fused but distinct (Fig. 1, B). Vein  $M_2$  of wing not discernible even after staining. Foretibial comb consisting of a row of five short, stout, curved chaetae minutely setose, backed by four small and one larger chaetae (Fig. 1, C). Spermathecae two, pyriform, equal (Fig. 1, D).

*Male*.—Wing  $0.7 \times 0.3$  mm. Color as in female but slightly darker. Genitalia: median ventral surface of ninth sternite produced into backward-pointing conical process bearing many large chaetae and terminating in two broad, pointed chaetae with strong basal tubercles. Basistyles short, broad, their apodemes slender, joining in flat-topped arch. Dististyles untapered to knob-like process on anterior surface beyond middle, thence continuing as slender, curved tip. Aedeagus wide, U-shaped, with two down-turned flanges and twin terminal valves (Fig. 1, L).

*Holotype* ( $\sigma$ ).—St. Augustine, Trinidad, B.W.I., August 10, 1957, reared from rotting leaves in cacao plantation. Deposited, with specimens of all other stages, in National Collection, Ottawa, No. 6729.

*Paratypes*.—Two larvae, one pupa, one  $\varphi$ , and associated exuviae, same data as holotype. Two larvae, one pupa, three  $\sigma$ , and associated exuviae, from rotting cacao leaves, Maracas Valley, Trinidad, B.W.I., July 14, 1957. One larva, one  $\sigma$ , and associated exuviae, from rotting cacao leaves, Las Hermanas plantation near San Rafael, Trinidad, B.W.I., August 7, 1957. One  $\sigma$  taken at light, St. Augustine, Trinidad, July 1, 1957. In personal collection.

The male genitalia are so unusual in this species that the male is designated holotype rather than the larva; no other known species of *Proforcipomyia* has the knob-like process on the anterior surface of the dististyles, nor the chaetigerous cone on the ninth sternite. The chaetotaxy of the larva and the tubercles and prothoracic horns of the pupa are specifically distinct, but not unusual.

This is the commonest species of *Proforcipomyia* breeding in the mat of dead leaves in cacao plantations in Trinidad during the early part of the rainy season. Females were never taken in cacao flowers, but a newly emerged female kept in a vial with fresh flowers was found to bear some pollen grains on the thorax some hours after imprisonment, although she showed no interest in the flowers while under actual observation. It would appear, therefore, that *mortuifolii* may be one of the pollinating agents of cacao.

*Forcipomyia (Proforcipomyia) setigera* Saunders, new species

*Larva*.—Length 2.55 mm. Color: pinkish-brown subcutaneous pattern throughout body, stronger on thorax. Head light brown, profile strongly scalloped; *p* hairs small, rod-like, *q* hairs longer, with swollen tip; *a* multiple, five-partite chaeta on each side of head (Fig. 2, B). *a* hairs of body in form of very small, tapering chaetae; dorsolaterals short, dark, sharply bent, serrate on outer border; four pairs fine lateral hairs per segment. Last body segment bearing double dorsal tubercle with setae longer than entire body; flanking it are two pairs of small multiple chaetae (Fig. 2, A, C).

*Pupa*.—Length 2.4 mm. Exuviae pale yellowish. Three tubercles on median sclerite of head, none on laterals; three pairs small tubercles on thorax, anterior pair setigerous; only minute setae on abdomen; terminal

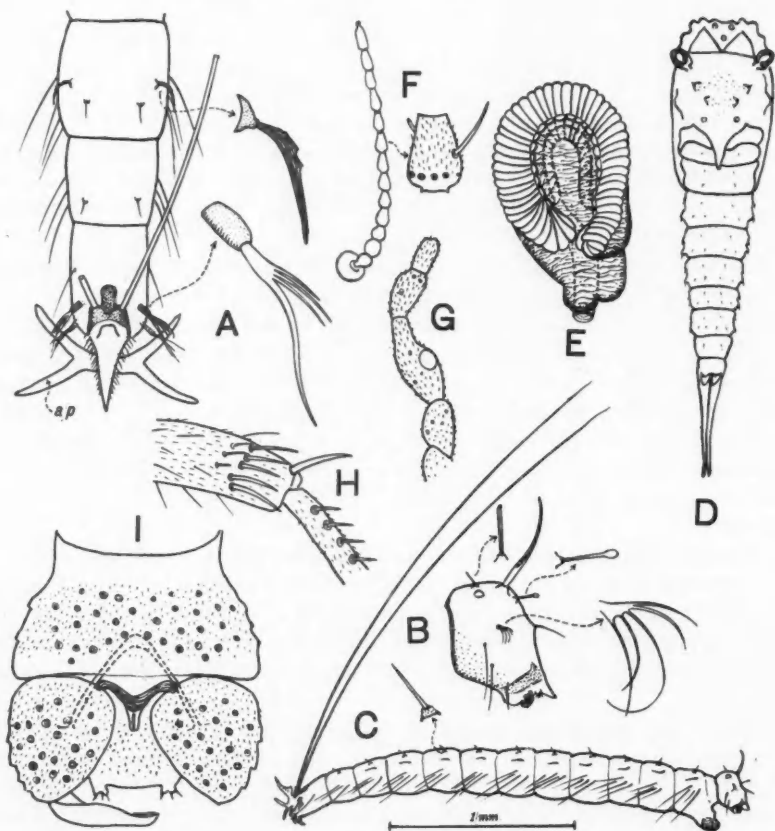


FIG. 2. *Forcipomyia (Proforcipomyia) setigera*. A, last three segments of larva; B, larval head; C, entire larva; D, pupa; E, pupal prothoracic horn; F, female antenna; G, female palp; H, foretibial comb of female; I, male genitalia. *ap*, anal papillae.

processes in both sexes more than four times as long as basal part of segment (Fig. 2, D). Prothoracic horn with oval closed circlet of about 55 spiracular papillae occupying most of the horn (Fig. 2, E).

*Female*.—T.R. 1.8. Wing  $0.7 \times 0.33$  mm. Ground color reddish brown, thorax and abdomen dark grey, uniform; legs pale, testaceous; halteres white. Antennal segment 4 widest, globular; subsequent segments becoming narrower and more elongate, with no abrupt change between 10 and 11; sensory hairs shorter than length of segment (Fig. 2, F). Palps with circular sensorium on swollen part of 3, segments 4 and 5 distinct, not fused (Fig. 2, G). Foretibial comb consisting of a row of five simple curved chaetae all shorter than tibial spur, backed by very small chaetae (Fig. 2, H). Spermathecae subequal, pyriform.

*Male*.—Wing  $0.85 \times 0.3$  mm. Color as in female but slightly darker. Genitalia: ninth sternite twice as wide as long; basistyles little longer than wide, joined by apodemes in pointed arch; dististyles stout, simple, spoon-shaped distally; aedeagus small, dark, in very open V-shape, terminal valves comparatively long (Fig. 2, I).

*Holotype* (larva).—Maracas Valley, Trinidad, B.W.I., July 18, 1957, from rotting leaves in cacao plantation. Deposited, with specimens of all other stages, in National Collection, Ottawa, No. 6726.

*Paratypes*.—One ♀, one ♂, and associated pupal exuviae, same data as holotype. In personal collection.

Two other species with long larval tail setae are known: *phlebotomoides* Bangerter (1), from the European Alps, and *longispina* Saunders (7), from Brazil. In all three species there are characters which separate them from other *Proforcipomyia* species, notably the long tail setae, the compound hairs of the head and tail, the setigerous tubercles on the thorax of the pupa, and the attenuation of its terminal processes. Morphologically, *setigera* is close to *longispina* in many of these characters, but it is smaller and differs markedly in the pupal prothoracic horns which in *longispina* are discoidal and bear nearly 200 spiracular papillae. Breeding in the layers of rotting leaves in a cacao plantation, this may well be another of the pollinating agents, but it was impossible to prove it.

*Forcipomyia* (*Proforcipomyia*) *falcifera* Saunders, new species

*Larva*.—Length 2.3 mm. Color: strong pinkish-brown pigment pattern throughout body, lighter towards tail. Head pale yellow-brown; profile strongly flattened in two areas; *p* and *q* hairs spear-shaped (Fig. 3, B). *a* hairs of body segments spear-shaped, the swollen blade occupying nearly half the total length; last pair, with fused basal tubercles, long, reaching well beyond tip of cauda; dorsolateral hairs very short, like curved knife with serrated edge; four fine lateral setae per segment (Fig. 3, A). Prothoracic pseudopod with longer spinules than usual and bearing two slender terminal hooklets (Fig. 3, C).

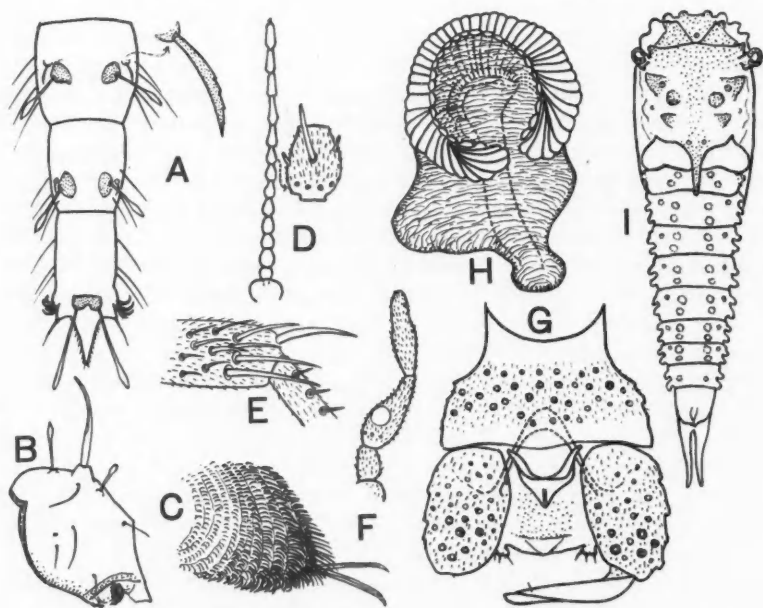


FIG. 3. *Forcipomyia* (*Proforcipomyia*) *falcifera*. A, last three segments of larva; B, larval head; C, prothoracic pseudopod; D, female antenna; E, foretibial comb of female; F, female palp; G, male genitalia; H, pupal prothoracic horn; I, pupa.

**Pupa.**—Length 2.3 mm. Exuviae pale yellowish. An excessively tuberculate pupa, with three tubercles on median sclerite of head, one each on laterals; on thorax one in front of each prothoracic horn, on dorsum three large pairs and three very small pairs, one of which is on base of anterior large pair; abdominal segments with three pairs of tubercles on dorsum and two on sides. Terminal processes moderately elongate (Fig. 3, I). Prothoracic horns broad basally, bearing prominent heel on posterior aspect; spiracular papillae in complete circlet with about 50 papillae in lower palisade (Fig. 3, H).

**Female.**—T.R. 1.6. Wing  $0.7 \times 0.3$  mm. Color: generally dark grey, but with broad median stripe on thorax brownish yellow; chaetae on thorax and legs golden; halteres grey. Antennal flagellar segments slightly elongate basally, becoming gradually longer to more abrupt increase at 11; one hyaline sensory hair shorter than segment, two others very small (Fig. 3, D). Palpal segments 4 and 5 completely fused (Fig. 3, F). Foretibial comb consisting of a row of slender simple chaetae, the longest of which exceeds the tibial spur, backed by two rows of successively shorter chaetae (Fig. 3, E). Spermathecae subspherical.

**Male.**—Wing  $0.9 \times 0.3$  mm. Coloration as in female but slightly darker. Genitalia conforming closely to usual pattern in *Proforcipomyia*: pronounced emarginate neck on ninth sternite; basistyles nearly twice as long as wide,

joined internally by apodemes in pointed arch with wide membranous top; dististyles stout, tapering to fine, spoon-like tip; aedeagus shield-shaped, deeply emarginate (Fig. 3, G).

*Holotype* (larva).—Las Hermanas cacao plantation near San Rafael, Trinidad, B.W.I., August 7, 1957, from epiphytic bromeliad. Deposited in National Collection, Ottawa, No. 6727, together with specimens of all other stages.

*Paratypes*.—One ♂, one ♀, and associated pupal exuviae, in personal collection.

This species has the closest affinities with *Forcipomyia* (*Proforcipomyia*) *bromeliae* Saunders (7); the two are the only known species of *Proforcipomyia* with terminal hooklets on the prothoracic pseudopod, and both have the complete circlet of spiracular papillae on the pupal respiratory horn, although *falcifera* has a more pronounced heel at the back. However, the most distinctive feature of all is the highly tuberculate condition of the pupa, since *Proforcipomyia* pupae are usually very smooth. Breeding in a bromeliad on a shade tree in the heart of a cacao plantation, this might well be a pollinator, but the fact could not be proved.

I can describe and figure two actual pollinators, but having only females I do not name them. They fit Macfie's description of *quasi-ingrami* (4), but so do all the species described above, if females only are considered.

*Forcipomyia* (*Proforcipomyia*) species A

*Female*.—T.R. 1.8. Wing  $0.7 \times 0.3$  mm and  $0.95 \times 0.4$  mm. The usual dull grey-brown color without distinctive markings. Antennae with globular basal segments, becoming slightly elongate at 7 and abruptly longer at 11; two slender hyaline sensory hairs of same length as segment (Fig. 4, C). Foretibial comb with a row of four slender chaetae shorter than tibial spur, backed by smaller chaetae in triangular arrangement (Fig. 4, A). Fourth and fifth palpal segments fused but showing faint separation line; circular sensorium on moderately swollen third segment (Fig. 4, B). Spermathecae pyriform, unequal in size.

Described from one larger and two smaller females, all of the same species, taken in a single cacao flower at 3:30 p.m., August 29, 1957, on the estate of Mr. Branch, St. John's, Grenada, B.W.I.

*Forcipomyia* (*Proforcipomyia*) species B

This female was not taken actually in a flower, but was resting on a bud; the fact that it bore pollen grains on the thorax may be interpreted as proof that it is a pollinating agent.

*Female*.—T.R. 1.2. Wing  $0.98 \times 0.43$  mm. Color dull grey-brown, without distinctive markings. Antennal flagellar segments elongate even at base, becoming longer and with an abrupt change at 11; hyaline sensory hairs three, fine, shorter than segment (Fig. 4, F). Palps slender, third segments only slightly swollen, with small circular sensorium at less than

half its length; complete articulation between fourth and fifth segments (Fig. 4, E). Foretibial comb a compact row of six slender chaetae, none as long as tibial spur, with no smaller chaetae above (Fig. 4, D). Spermathecae pyriform, subequal.

Cacao finca "Theobroma" near Siquirres, Costa Rica, September 5, 1957.

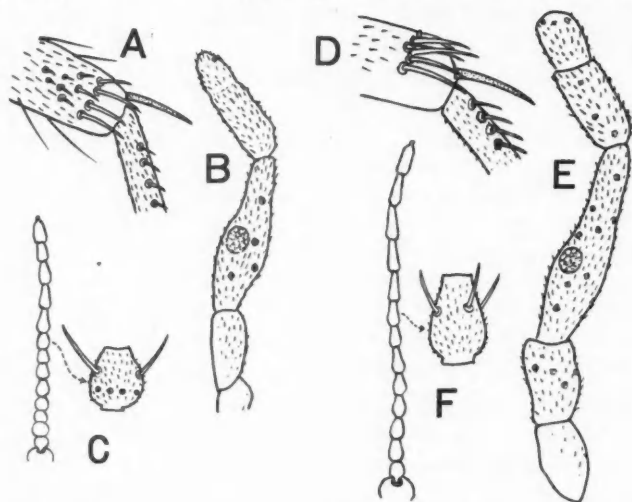


FIG. 4. A,B,C, *Forcipomyia* (*Proforcipomyia*) sp. A; D,E,F, *Forcipomyia* (*Proforcipomyia*) sp. B. A,D, foretibial comb; B,E, female palp; C,F, female antenna.

#### SUBGENUS *Thyridomyia*

Females are dull, dark-brown, hairy little flies; all species so far known in their early stages have a strong orange internal pigmentation which persists as an orange ground color in membranous areas of the adults. The hind metatarsus is very long, the tarsal ratio extending from 2.3 to 3.4. The flagellar segments of the antennae are globular, even compressed longitudinally and elongating moderately at 11. The palps are variable, with the last two segments never fused. The second radial cell of the wing is often elongate, leading to confusion with the anomalous genus *Lasiohelea*. Spermathecae single.

Males are usually somewhat darker than the females. Their genitalia are distinguished by the form of the basistylar apodemes, which project forward as a spur, bearing a triangular plate or slender process extending mediad; there may also be club-like parameres directed backward, in which case there is possible confusion with *Synthyridomyia*. A large excavation of the ninth sternite in the form of a Moorish arch usually characterizes the subgenus, but there are some species with only a shallow excavation, as in the case of the species described below, and Macfie's *nana* (4); this again resembles *Synthyridomyia*. The aedeagus is large and complicated, but contributes little to the subgeneric diagnosis.

Larvae are more or less orange-colored in all species known so far. The position and nature of the antenna is the most characteristic feature of the subgenus: moved forward on to the front of the head, it is reduced to a flat disc on a low sockle, with an extremely small flagellum in the membrane outside the disc. The vertex of the head bears two or three rounded swellings. The body is variously tuberculate and spiny, but there are no spear hairs or others homologous with the *a* hairs of most other subgenera. The prothoracic pseudopod is a spinulose cushion bearing terminal hooklets in two groups; in one exceptional species (as yet unnamed) the pseudopod is cleft as in the subgenus *Forcipomyia*.

Pupae are less distinctive, bearing small or large tubercles on the head, thorax, and abdomen. The prothoracic horns furnish the best means of identification, being always broad, rather spade-shaped, with a row of complex spiracular papillae across the rounded extremity. The male sexual processes are ventral, and the larval skin is not retained on the tail of the pupa.

One new species is described, as an example of the subgenus. It was breeding on a mossy deadfall in a cacao plantation, and might well be a pollinator although this could not be proved.

*Forcipomyia* (*Thyridomyia*) ***nodosa*** Saunders, new species

*Larva*.—Length 1.9 mm. Color brownish red. Head pale with dusky pigmentation on vertex and front. Vertex with two pairs large swollen ridges within frontal sutures, a third, smaller pair outside; an unpaired median swelling on front below *p* hairs. Antennae below middle of front, on low conical sockle, the usual flat discoidal structure leaving space in the membrane outside for a minute flagellum. *p* hairs stout, torch-like; *q* hairs very small, with four-pointed tip. No ocular seta (Fig. 5, H). Body segments each with a dorsal and a laterodorsal pair of conical tubercles, conspicuous, coarsely shagreened, tipped by a minute rosette-like chaeta; penultimate segment bearing two pairs of dorsal tubercles, last segment none; a single pair of ventrolateral setae on each segment (Fig. 5, G). Prothoracic pseudopod a setulose cushion armed with about 10 pairs dark curved hooklets.

*Pupa*.—Length 1.5 mm. Color bright orange-red, with pale prothoracic horns. Tuberculate condition of larva continued in pupa: median sclerite of head with three tubercles, the anterior pair greatly enlarged; one tubercle on lateral sclerites; thorax with five pairs, arranged as shown in Fig. 5, I. Abdominal segments with prominent median pair of tubercles, small laterodorsals, prominent laterals; all tubercles coarsely shagreened; terminal processes turned out at right angles to body axis, but larval skin not retained despite this. Prothoracic horn almost parallel-sided with broad base; row of about 15 complex spiracular papillae curving over rounded extremity (Fig. 5, J).

*Female*.—T.R. 3.4. Wing  $0.6 \times 0.3$  mm. Ground color red-brown showing between darker sclerites; wing hairs dusky; halteres white. Antenna with basal flagellar segments compressed, becoming as long as wide at 9 and 10,

with marked increase in size and length at 11; hyaline sensory hairs short, curved (Fig. 5, B). Second radial cell of wing open, elongate, nearly twice as long as first (Fig. 5, A). Palps short, stout, third segment with practically no neck beyond swollen portion, sensorium an unconfined group of capitate sensilla near end of segment; fourth and fifth segments globular, distinct (Fig. 5, D). Spermatheca subspherical, tapering to duct (Fig. 5, C). Spermatheca subspherical, tapering to duct (Fig. 5, C).

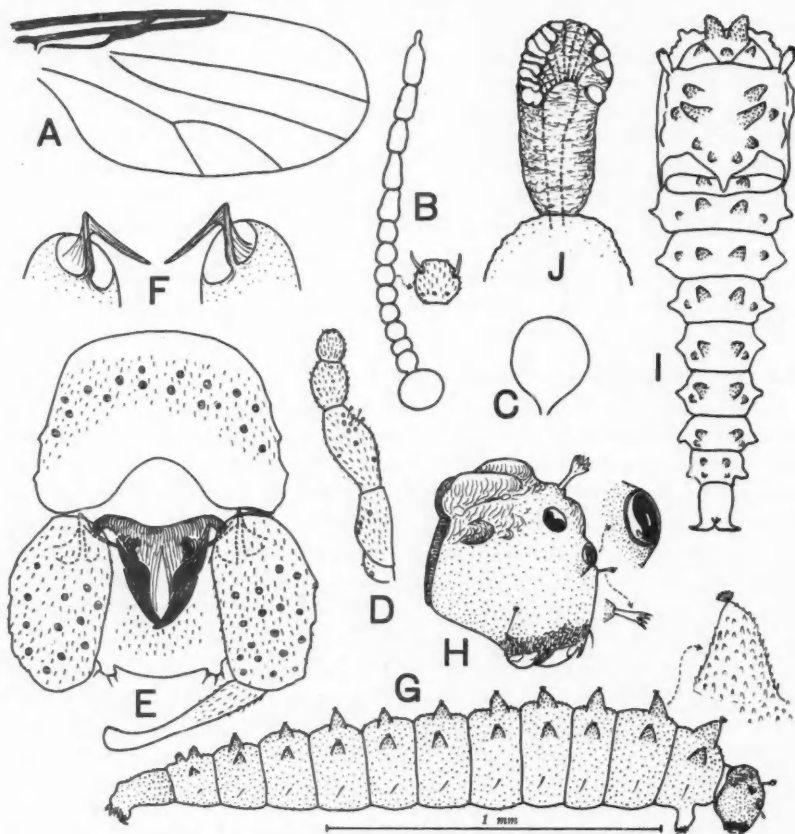


FIG. 5. *Forcipomyia* (*Thyridomyia*) *nodosa*. A, female wing venation; B, female antenna; C, outline of spermatheca; D, female palp; E, male genitalia; F, basistylar apodemes in dorsal aspect; G, entire larva; H, larval head; I, pupa; J, pupal prothoracic horn.

*Male*.—Wing  $0.8 \times 0.25$  mm. Color as in female, but wings thinner-scaled, iridescent. Genitalia: ninth sternite broader than long, without anterior neck, posterior excavation rounded, extending forward one third length of segment; basistyles twice as long as wide, apodemes a short spur directed forward, bearing thin inner processes which represent the hypotenuse of the

usual triangular plate (Fig. 5, F); dististyles swollen at tip, nearly as long as basistyles; aedeagus with strong, curved, backward-directed blades bearing flange-like prominence near base (Fig. 5, E).

*Holotype* (larva).—Finca "Theobroma" near Siquirres, Costa Rica, June 15, 1956, on mossy deadfall in deep shade. Deposited, with specimens of all other stages, in National Collection, Ottawa, No. 6728.

*Paratypes*.—One larva, two ♂♂, four ♀♀, seven pupal exuviae, same data as holotype, in personal collection.

Macfie's *nana* (4) is the only other species described from the neotropics (Brazil and Trinidad), and his figure of the male genitalia shows complete triangular plates on the apodemes, longer basistyles, unswollen dististyles, and a different aedeagus. Because of the elongated second radial cell of the wing, he placed it in the genus *Lasiohelea*, but it is now becoming evident that the character is common to several groups of midges whose early stages show them to be subgenera of *Forcipomyia*.

### Methods of Collection

#### Adults

To catch midges visiting cacao flowers and transferring pollen, the investigator must be resigned to patient and prolonged observation. All workers agree that they are most active in the morning from 8:30 to 11:00 a.m., and again in the afternoon after 3:00 p.m.; presumably they rest in the heat of the day. When a midge is seen to alight on a staminode, it is advisable to allow it to pass into the petal hood before attempting to enclose it, for it can escape with remarkable agility. Capture is effected by swiftly corking the flower into a vial of 1 in. diameter. The only alternative is to place the vial over one flower after another in the hope that an unseen midge is within; the opening of the vial must be pressed firmly against the flower cushion, but there still follows the problem of corking without losing the specimen. On the only occasion that I enclosed a midge in this manner, it discovered a gap and departed with astonishing celerity.

By sweeping through cacao foliage and around flowers on trunk and branches with an insect net, a sample of the population can be collected, but if *Proforcipomyia* females are present there is no indication that they are pollinators unless some pollen adheres to the body, an unlikely condition after the rough treatment they receive in the net.

#### Early Stages

*Forcipomyia* larvae must have moist situations where bacteria, molds, and yeasts flourish, and *Proforcipomyia* larvae want it wetter than most other subgenera. Carter (3) described *ingrami* from larvae swarming on the walls of tree rot-holes; the type species, *wirthi* (7), was just as numerous on the algae and stones around a hot spring in California. In the neotropics, bromeliads often harbor larvae in the lower leaf axils where detritus accumulates but is still wet; other plants with water-holding leaf axils such as aroids,

*Dracaena*, *Pandanus*, even banana, may occasionally serve as breeding places for *Proforcipomyia*. However, more species are recovered from the ground, on dead leaves lying partly in water, in moss growing on soil or wood. In cacao plantations the most fruitful source seems to be the mat of dead leaves where these accumulate in sufficient depth to remain permanently wet. Whereas visual examination is adequate for most of the situations mentioned above, the leaf mat is so extensive and the larvae so few that they could never be found there visually. Therefore, recourse must be had to the Berlese funnel, a well-known device for extracting organisms from moist material. Essentially the apparatus consists of a fine copper screen covering a funnel, supported in some manner so that the mouth of the funnel has about an inch of clearance. Dead leaves, leaf mold, moss, or any moist material suspected of harboring *Forcipomyia* larvae is placed in a thin layer on the screen and a hot electric light in reflector is suspended above. As the material dries, the larvae and even pupae work down for moisture, penetrate the screen, and descend the funnel, arriving in a dish provided for their reception. Most Berlese workers collect directly into alcohol, but *Forcipomyia* larvae are needed alive for rearing, so a shallow dish is prepared containing a pad of

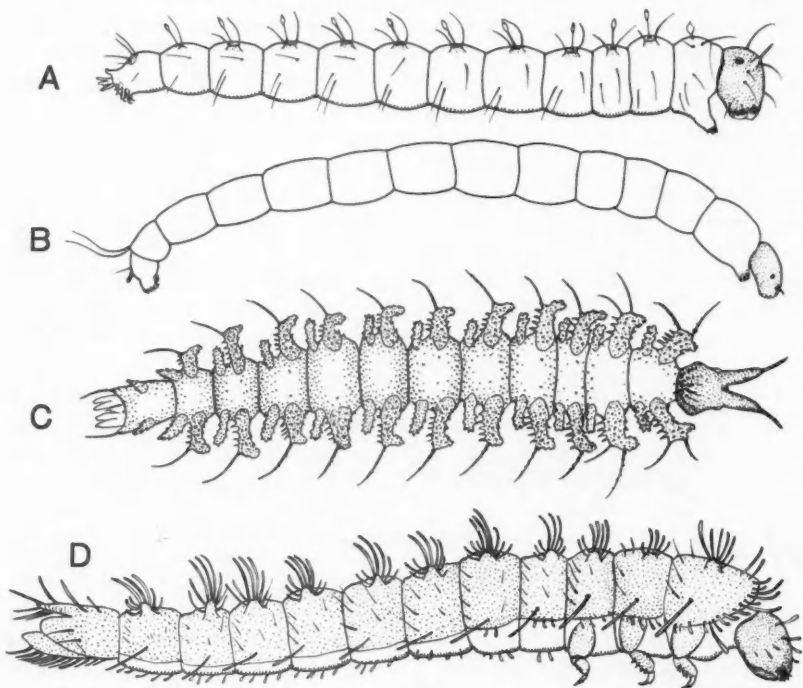


FIG. 6. Some larvae recoverable from rotting cacao leaves in Trinidad which might be confused with pollinating *Forcipomyia* larvae. A, *Forcipomyia* (*Forcipomyia*) *raleighi*; B, chironomid; C, *Atrichopogon tuberculatus*; D, coleopterous larva.

moist cotton covered with a filter paper. I use the bottoms of plastic tumblers, which are unbreakable and can be placed under the dissecting microscope for examination. Figure 6 shows some of the larvae which might conceivably be confused with *Proforcipomyia* or *Thyridomyia* larvae; all of these occurred quite commonly in rotting leaves in Trinidad. There will also be predaceous larvae of Diptera and Coleoptera, mites and spiders, waiting to consume the desired larvae as they descend; these must be removed with forceps during the drying process.

If a Berlese funnel is to be used permanently in one location, it may measure a foot or more at screen level and may have a retaining rim several inches high, which helps to hold the large cacao leaves. The funnel is normally of metal and must have a fairly steep pitch in order that larvae will not lodge and die. On the other hand, Fig. 7 shows a Berlese funnel designed to fold flat and pack away in baggage—two of these have served me well in many countries. One improvement I would suggest would be to make the legs themselves serve as locking screws, but some means would have to be provided

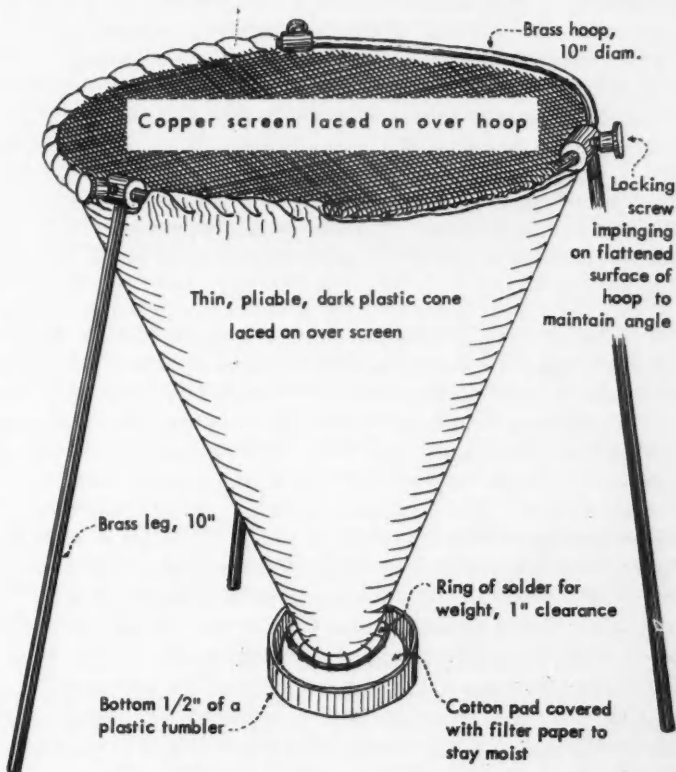


FIG. 7. Diagram of a folding Berlese funnel.

to hold the leg firmly while screwing it in. In field work where electric light is not available, the funnel can be put out in the sun: tropical sunlight soon dries materials, but it will dry the moist dish below if it can reach it.

*Thyridomyia* larvae occur in moss, mostly on rotting wood; tufts, scrapings, or slices of such material, collected in pliofilm bags and put through the funnel, often yield interesting larvae. I also recovered a family of very young *Thyridomyia* larvae from a thin growth of moss on wet clay surfaces such as the walls of drainage ditches and exposed soil around the base of cacao trees in Trinidad, but failed to rear them. I have never recovered *Forcipomyia* larvae of any kind from leaf mold or the soil beneath the leaf cover, but the worker must keep an open mind and regard every moist place in the vicinity as a possible breeding place.

### Rearing

Larvae are best removed from the dishes with a fine, moistened camel's-hair brush. They will usually continue their development in confinement on a small portion of their native medium in a vial, be it rotting leaf, rotting wood, mossy wood, or soil. The material should not be too plentiful, for it is necessary to recover the pupal exuviae after emergence of the adults (characters of the pupa are more readily seen on exuviae than on the whole pupa). In northern countries larvae of most species will go into diapause in the autumn, even though brought in to room temperature and subjected to artificially lengthened daylight. The diapause can sometimes be broken by placing the rearing vials directly on ice in the refrigerator for 3 or 4 months; the alternative is to bury them in a container outside until spring. In the tropics it is not yet known whether a diapause occurs during the adverse conditions of dry seasons.

### Rearing on Cultures

Examination of stomach contents reveals microorganisms of many kinds as the true food of the larvae, rather than the wood, leaves, or moss on which they are found. It seemed reasonable, therefore, that if these molds, yeasts, bacteria, etc. could be grown on artificial culture media, a plentiful supply of food would be more readily available than under natural conditions. Trial cultures in the north gave encouraging results, so the method was tested and adopted in the tropics. The technique now employed is to pour thick, firm, malt-agar plates in petri dishes; the agar should be 4% in water in place of the usual 2%, the malt extract 1% instead of 2%. Strict sterilization of medium and dishes is not necessary if inoculation is done within a few hours of cooling. The inoculum consists of scrapings taken from rotting leaves into a minimum of water, or water pressed from moss or moss-grown wood; the object is to get the organisms from the natural environment of the larvae to be reared. Within a couple of days a thick growth of fungal hyphae usually develops; when this dies down or is wiped off, the culture settles down to a mixed growth of many different organisms, with a mottled brownish appearance, and is thereafter resistant to any further contamination.

Rotting leaves contain bacteria with strongly liquifying effect on the agar, resulting in a very wet surface on the culture. *Proforcipomyia* larvae do not seem to mind this, but it may be deleterious to larvae of some other subgenera. Tests have been made with bacterial suppressives, and traces of streptomycin have been found effective when added to the original medium, without affecting the growth of the larvae themselves.

On these cultures larvae of several subgenera of *Forcipomyia* have been reared to maturity, even from very early instars, and often when they failed to develop on their own natural medium. As a safeguard, I usually plant an island of the natural medium in the center of the culture; larvae of some species prefer it, others ignore it.

It is advisable to transfer pupae to moist filter paper in a vial so that the adults may be easily captured after emergence.

A logical extension of the culture technique would be to develop a medium on which females would oviposit. Little has been done with this owing to lack of specimens of cacao pollinators with which to experiment. However, a gravid female of *Forcipomyia* (*Forcipomyia*) was taken in course of sweeping and introduced into a culture; next morning she was nearly dead, but she had deposited a pile of 112 eggs. In 3 days the eggs hatched and larvae spread all over the plate, but they failed to develop. Their death may have been because they had a special environmental preference and something was lacking in the culture, or it may have been due to a toxic culture. After about 6 weeks some cultures seemed to develop a toxicity; larvae which would normally have lived there died soon after introduction. On the other hand, in an early experiment in Canada, eggs must have been introduced with the inoculum, for a family of first-stage larvae appeared and thrived; within 3 weeks they had pupated and emerged—a phenomenal rate of growth.

It would seem, therefore, that a large-scale modification of this technique might be developed to raise *Forcipomyia* midges in plantations where natural pollination is inadequate. It also has application to related fields of study, for larvae of cecidomyiids, mycetophylids, some chironomids, and other dipterous larvae flourish on the cultures. I hopefully recommend it to workers who are in a position to obtain females of *Pterobosca* from the wings of dragonflies and other insects—dozens of species have been described, but no one has ever seen a male or the early stages.

Another possible use for culture-rearing would be the addition of radioactive isotopes to the medium, thus producing tagged adults which could be released at various distances from flowering cacao and picked up again by Geiger counter.

### Killing, Preserving, Studying

Reared adults must be given time to harden and assume their full coloration, then a male and a female should be killed dry and color notes recorded. All others can be killed by touching with an alcohol-wet brush and preserved in 75% alcohol. After their color is recorded, living larvae should be killed by plunging into water just boiled; this extends pseudopods and anal papillae,

the expansion often continuing in the preserving alcohol and frequently causing a welcome evacuation of part of the intestine. If facilities for heating water are not available under field conditions, any fixative containing acetic acid, such as Carnoy's, will extend most larvae. Pupae should not receive this treatment, for expansion will distort them. Pupal exuviae need manipulation under the dissecting microscope to expel air and allow them to sink.

Various tubes are employed by different workers for storage: I use microvials 5 mm  $\times$  50 mm; 10 of them can be kept in a 1 in.  $\times$  3 in. vial. Data are written in waterproof ink on a narrow strip of paper, on both sides if necessary, inserted, and the tube plugged with cotton. For permanent storage the microvials are inverted in the alcohol-filled larger tube, cushioned with a layer of cotton; they can then dry out only when the surrounding alcohol evaporates right to the bottom.

Examination, measuring, and drawing are all done with the "sloping cover slip" technique. Half of a cover slip 18 mm square is supported at one end of a low bar of paraffin wax on a slide. Water, alcohol, or clearing oil can be introduced and the specimen inserted from the side. For handling such small specimens an indispensable instrument is the flattened needle, made by hammering flat the last 8 mm of a No. 1 or No. 2 insect pin; the shaft is shortened and forced into a slender wooden handle; the blade is bent to an angle of about 45°. Objects can be lifted out of a dish with this, inserted under the sloping cover slip, and worked down the wedge-shaped space until they jam slightly. Final adjustment can often be done by moving the cover slip so that the specimen beneath rolls in the desired direction. When exact orientation is achieved under the dissecting microscope, the slide can be transferred to a standard microscope for examination and drawing by means of camera lucida, squared field, or bioscope projection.

It will usually be found that dehydration in 95% alcohol and clearing in oil permit adequate examination of all features, but male genitalia sometimes need preliminary clearing in KOH. I use creosote oil rather than terpineol because Light Green stain will dissolve in it; a wing from each sex goes from 95% alcohol to the oil stain to bring out venation. All drawing is done before permanent mounting, because once in balsam only one aspect of a specimen can be seen; moreover there may be collapse of appendages, spermathecae, or whole specimens.

The techniques described above will be routine procedure to all seasoned workers, but they are presented for the guidance of students and beginners in this field.

### **Suggestions for Future Work on Cacao Pollinators**

A great deal remains to be discovered about these most important insects.

1. The breeding places of the species involved in any one locality must be discovered, and all stages described.
2. How many generations are there in a year? Do they have a seasonal fluctuation? Where are they during the dry season?

3. Are they actually feeding when they visit cacao flowers and probe the staminodes? If so, do they go up into the petal hood for more or different food? On what do they feed when the cacao is not in flower?

4. Will females visit flowers directly after emergence (to demonstrate their potentialities) or must they mate first?

5. How far will they travel to and from their breeding grounds?

6. Are any species specially effective or more easily raised, and therefore worthy of distribution?

7. Can they be adapted to large-scale culture-breeding? If not, what can be done to improve their natural breeding sites, where more are desired?

### Acknowledgments

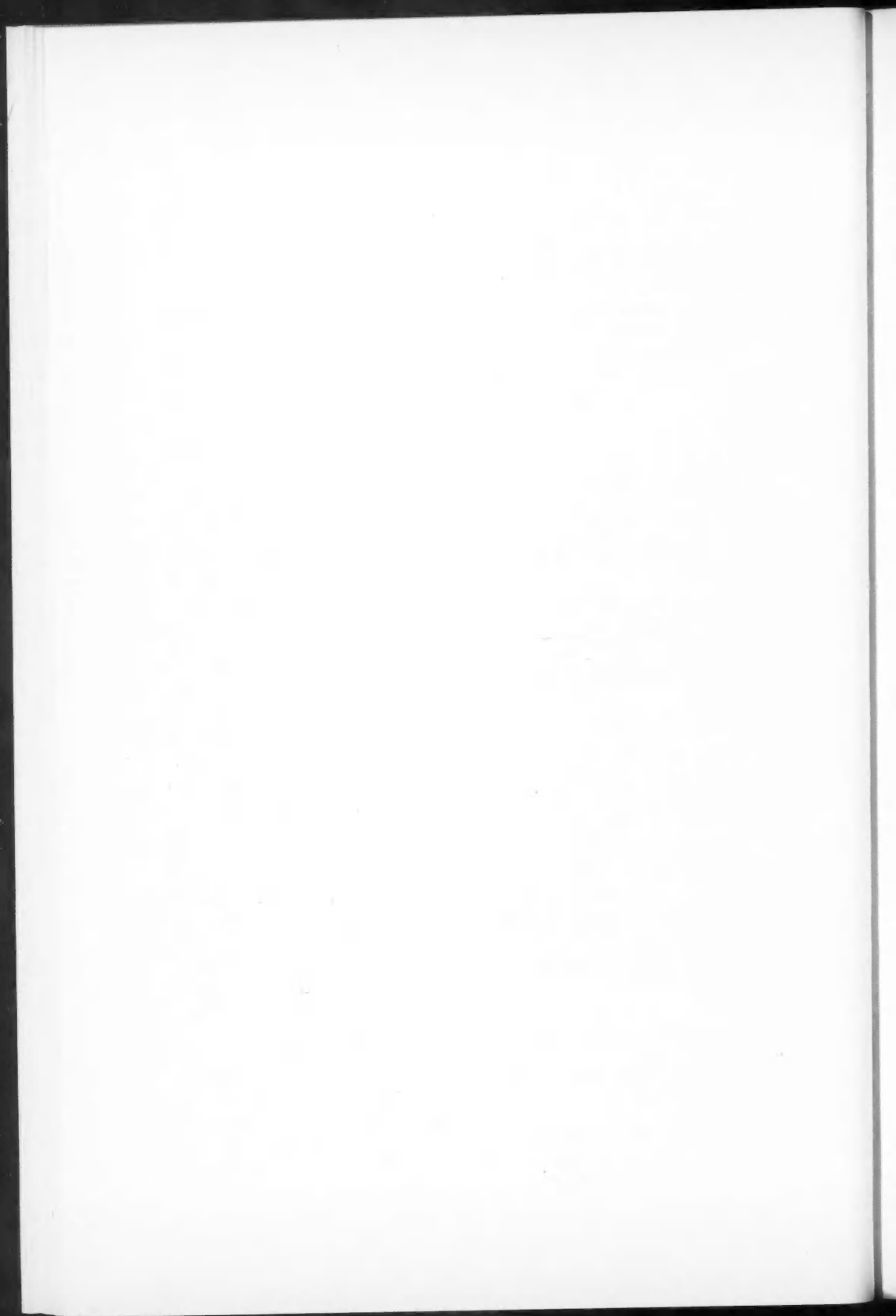
I wish to acknowledge the contribution of the Banco de Costa Rica, whose Board financed a month's visit to that country in 1956; and in particular to thank Mr. George E. Bowman of San José, whose active assistance made the investigation possible.

A much larger sum was contributed by the American Cocoa Research Institute, Washington, in 1957, permitting a 3-month study in Trinidad and Costa Rica.

To the authorities of the Imperial College of Tropical Agriculture, St. Augustine, Trinidad, I extend my sincere thanks for laboratory facilities, and I am grateful to Mr. Perryman and Mr. Cruikshank of the Cocoa Research Station, Mt. Horne, Grenada, for a conducted tour of cacao plantations in that island.

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# SOME OBSERVATIONS ON CRISTISPIRA IN THE CRYSTALLINE STYLE OF SAXIDOMUS GIGANTEUS DESHAYES AND IN THAT OF SOME OTHER LAMELLIBRANCHIATA<sup>1</sup>

C. BERKELEY

## Abstract

The occurrence of a spirochaete of the genus *Cristispira* in the crystalline style of *Saxidomus giganteus* Deshayes is recorded. The distribution of the organisms in the style, and the possible reasons for their absence in the anterior region of it, are discussed. It is found to be a facultative anaerobe. Observations are recorded on the infection of newly regenerated styles, and occurrences of *Cristispira* in styles of species of Lamellibranchiata other than *Saxidomus giganteus*, and of their absence elsewhere, are summarized.

## Introduction

The normal presence of large spirochaete organisms living in the digestive tract, and especially in the crystalline style, of Lamellibranchiata has been known for many years. The first record appears to have been that of Certes (4), who observed it in the stomach of *Ostrea edulis*. The genus *Cristispira* was established by Gross (8) to contain such forms characterized by their habitat, their large size, and the possession of a flattened ridge or "crest" running the length of the body and making several turns around it, known as the "crista". Most of the recorded observations on these forms have been made on oysters, but they have been found from time to time in other lamellibranchs. Dimitroff (5) summarizes the early literature of the subject and there seems to be little subsequent to his paper. Investigations have hitherto been directed almost exclusively to consideration of the distribution of the organisms among the various hosts, their detailed morphology and staining reactions in their bearing on attempts at classification, and efforts to cultivate them in artificial media.

The observation that *Cristispira* occurs in enormous numbers in the style of *Saxidomus giganteus*, one of the commonest "clams" inhabiting beaches of gravel and sand off the coasts of British Columbia, led to an effort to follow other points of interest in connection with these microorganisms.

## Extraction of the Crystalline Style

The crystalline style has been found to be a normal component of the digestive system of all lamellibranchs which have been carefully examined, provided they have been freshly removed from their natural habitat or have been maintained under equivalent environmental conditions. It is dissolved more or less rapidly, however, if the normal physiological activities of the mollusc are disturbed, and is not regenerated until these are restored. The glandular mechanism whereby the style is secreted may be present in either

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of two forms, one being merely a groove in the wall of the digestive tract, so that the style is fully exposed to, and is readily dissolved by, the more alkaline gastric fluid; the other in which the style is secreted in a narrow diverticulum, which it fits tightly and only one end of it is extruded into the stomach. In the latter case only this extruded end is exposed to the gastric fluid, and the style is dissolved relatively slowly. Yonge (12) discusses this matter. *Saxidomus giganteus* has a well-defined diverticulum. The style is readily extracted from a healthy animal if an incision is made into the stomach through the body wall just adjacent to the hinge of the shell. Slight pressure on the body causes the style to project through the incision. All styles used in the present experiments were extracted in this way.

### *Cristispira* in the Style

When clams were dug from well-aerated beds the styles of *Saxidomus giganteus* invariably harbor a large population of *Cristispira*. In only a few cases, in which the clams have been taken from muddy beds containing much decaying vegetable matter, have they been absent. The organisms are easily visible in a piece of crushed style at a low magnification (16 mm objective,  $\times 10$  eyepiece). They move in a characteristically undulatory manner in all directions within the style and, at first sight, seem to be more or less uniformly distributed in it. Since, however, the style is kept constantly revolving when the clam is feeding and is, simultaneously, pressed against the gastric shield, thereby grinding the food and mixing it with the digestive ferments of the style, it seemed unlikely that the *Cristispira* population could be maintained subjected to this grinding and digesting process. A systematic examination of a number of styles was therefore undertaken with a view to elucidating this point. Careful search in the tassel of ground food material which is always attached to the exposed end of the style failed to disclose any *Cristispira* or any fragments which could be recognized as parts of them. A number of styles were then cut transversely into a series of segments, starting at the narrow, recently secreted, end, and each segment examined for *Cristispira*. They were found in most segments throughout the length until the grinding end of the style was approached. None, or extremely few, were ever found in fully developed styles (about 50 mm long) within two or three millimeters of the region immediately behind the beginning of the frayed terminations. It would seem, therefore, that the organisms are sensitive to some external adverse influence when they reach this point and retreat from it.

Two possibilities as to what might be the nature of this influence suggested themselves. The first arose from an unpublished observation made by Dr. Rittenberg, of the Bacteriology Department of the University of Southern California, who has studied the *Cristispira* of the style of the "Pismo clam" (*Tivela stultorum*), a clam nearly allied to *Saxidomus giganteus*. Dr. Rittenberg found that if a solution of styles in freshly boiled and cooled sea water was

maintained at a low temperature in a completely filled and sealed vessel the *Cristispira* remained alive and active for some days. If, however, a drop of the liquid was removed and exposed to air on an uncovered microscope slide motility ceased in a few minutes and they appeared to die. They survived a little longer, as judged by motility, if the drop was covered with a cover slip. From this Dr. Rittenberg concluded tentatively that *Cristispira* "is strictly anaerobic and apparently quite sensitive to Oxygen", surviving in the anaerobic environment set up in the sealed vessel, but dying on exposure to air. Dr. Rittenberg has kindly allowed me to record his observation and inference. I have confirmed his observation, but cannot agree as to the inference. On several occasions solutions of styles (averaging about one style per 1 cc) were made up in sea water, freshly boiled out and cooled in a sealed vessel and maintained at a low temperature (4° to 8° C). The anterior few millimeters of the styles, including the whole of the food tassels, were discarded and the remainder carefully washed in changes of boiled and cooled water before use, in order to facilitate observation of the *Cristispira*. Complete solution of the styles results within the temperature range indicated in 12 to 15 hours (it is considerably more rapid at room temperature) and large numbers of *Cristispira* are present, actively swimming. If equal volumes of the solution were kept in (a) a wide-open tube, which was vigorously shaken at frequent intervals, and (b) a tightly sealed tube, under the same low temperature conditions, *Cristispira* in both tubes remained equally active for periods of days (up to at least eight) and no difference could be observed in survival periods. There was no evidence of marked growth of bacteria or of other foreign organisms in either tube. In both cases the *Cristispira* are seen to slow down and finally come to rest whilst under the microscope, but this seems to be due to rise in temperature. It seems, therefore, that *Cristispira* is a facultative, rather than an obligatory, anaerobe.

There is, however, some ground for surmise that, though it is not sensitive to molecular oxygen, it may be influenced by the strongly oxidizing environment, which is known to exist at the grinding end of the style, where it is frayed and mixed with ground food material. Oxidation activity is brought about by a peroxidase contained in the material of the style, but only in association with a constituent, or constituents, of the food material which, together, established a dehydrogenase system (Berkeley (2)). To test the possibility that this oxidizing environment might have some adverse effect on *Cristispira* a solution of styles containing active organisms was divided between two tubes and the tasseled ends of some fresh styles added to one of them, the other serving as a blank. Both were kept at a temperature near 5° C. After 5 hours, comparison showed that, in the first, the population of *Cristispira* had considerably decreased (in some cases disintegration seems to take place after death, a condition well illustrated by Bosanquet (3, Figs. 21 and 22, in the *Cristispira* of the style of *Anodonta* sp.) and those still present were quite non-motile. It is noteworthy that no organisms which might conceivably have been acting as a predator on *Cristispira* were present in the

culture. In the check tube the population seemed to have remained constant, and active swimming was quite general. Three hours later the contrast was even more marked. Though it cannot be concluded from these experiments that the *Cristispira* population is repelled by the oxidizing environment, since it might result from the direct toxic activity of some substance, or substances, extracted from the food material, the possibility appears interesting and worthy of further investigation.

The only alternative explanation which suggests itself is that it might be connected with the considerable difference in pH between that of the style and of the gastric fluid to which the extruded end is exposed in the stomach, to which reference has already been made. It seems unlikely, however, that the pH within the intact style could be influenced by that of the stomach contents since the style substance has a marked buffering action. Solutions of the style of *Saxidomus* in sea water (pH 8 to 8.2) vary in pH between 5.6 and 5.8 and maintain that pH even if heavily diluted. Solution in sea water buffered to a pH lower than 5.6 takes place very slowly at low temperatures, and the organisms within the incompletely dissolved style and the surrounding water show no motility after 18 hours.

#### ***Cristispira* in Relation to the Regeneration of the Crystalline Style**

Crystalline styles are found in quite small specimens of *Saxidomus* (15 mm in long diameter, or less). These have the usual dense population of *Cristispira*. It would be interesting to examine still younger individuals with a view to finding how early the infection becomes established and thus throw light on its primary origin. It is hoped to do this later.

Meanwhile a series of experiments was undertaken to determine the rate of infection of newly regenerated styles. As already mentioned the style of *Saxidomus* disappears rapidly if its normal physiological conditions are disturbed. The simplest and, in my experience, most effective way of doing this is to maintain the animals under anaerobiosis, which is soon established by keeping them in sealed jars of sea water packed as tightly as possible. At temperatures varying from 15° to 20° C complete disappearance of the styles occurs in 3 days or so. Full-grown specimens, taken from a population with well-developed styles having the normal content of *Cristispira*, were treated in this way until samples from the jars showed no styles present, either by the routine method of extraction described in an earlier paragraph or by dissection. They were then transferred to a well-aerated and cool environment, usually a coarse-meshed sack suspended at, or near, the bottom of the sea from a float. In these circumstances complete, though somewhat thin and flaccid, styles had usually developed in 24 hours. In 48 hours they were almost normal in both respects. Extremely few, if any, *Cristispira* could be found in the styles at this early stage. Specimens were examined at frequent intervals, but it was not until about 16 days after returning the clams to the sea that any quantity was to be found and a week later before

populations were normal. Bosanquet (3) records a similar instance of the absence of *Cristispira* in the newly regenerated style of a species of *Anodonta* which, normally, is heavily infected.

No evidence was obtained as to the source of the infection. It is probable that *Cristispira* remains in the digestive tract after the style disappears—indeed previous workers have found it there in other Lamellibranchiata (Certes (4), Fantham (7), Gross (8), Dimitroff (5), in oysters; Gross (8), in *Pecten*; Schellack (11), Fantham (7), in *Anodonta*). I have, however, not yet succeeded in doing so in *Saxidomus* and the possibility remains that infection may take place from the sea.

### The Specific Identity of the *Cristispira* of *Saxidomus*

Seven species of *Cristispira*, all from *Crassostrea* (*Ostrea*) *virginica*, are described in some detail by Dimitroff (5). He found that the type species *C. balbianii* (Certes) occurred most commonly and regarded two others *C. pectinis* Gross (8) and *C. veneris* Dobell (6) as synonyms of *C. balbianii*. Dimitroff's descriptions are summarized by Bergey (1) and he includes in his list of species two others, *C. interrogationis* Gross and *C. pinnae* Gonder, but these are somewhat inadequately described, as also are a number of other alleged species in the older literature.

The characters on which classification has been based are mainly (a) body-length, (b) body-width, (c) distance between the apices of the waves, (d) depth of the waves, (e) number of turns of the spira around the body-length. The body-lengths of most of the *Cristispira* in the style of *Saxidomus* vary from 60  $\mu$  to 75  $\mu$  (as judged from living, or recently dead, unfixed, specimens), but individuals as short as 30  $\mu$  and intermediaries between this length and 60  $\mu$  also occur. The width varies from 1  $\mu$  to 1.5  $\mu$ . The amplitude of the waves, their depth, and the number of turns of the spira about the body have been found too variable for satisfactory determination. The ends of the body are rounded, one end more definitely than the other. The spira is well developed and is best seen in unstained specimens. It is particularly well defined in specimens killed in cultures by introducing food-tassels from the grinding ends of styles, as described in a previous paragraph. However, staining intra-vitam with neutral red shows it well. A number of methods of fixation and staining were tried. The best results were from films obtained by lightly smearing cover slips with fragments of styles, flaming rapidly without previous drying, and staining with Giemsa's stain for 1 hour.

The characters of the *Cristispira* of *Saxidomus*, so far as they are determined, agree most closely with descriptions of either *C. balbianii* or *C. modiolae*, which two species seem to come so close as to bring them within the probable limits of variation. This seems, indeed, to apply to the descriptions of many of the species given in the literature, and separation of valid species in the group seems likely to remain difficult until methods of artificial culture can be devised. In these circumstances it has been judged best to be non-committal as to the species dealt with in this paper.

### *Cristispira* in the Styles of Other Species of Lamellibranchiata

The styles of two species of Lamellibranchiata, other than *Saxidomus giganteus*, which are common on British Columbia coasts, *Paphia staminea* and *Crassostrea gigas*, have been examined for *Cristispira* with positive results; those of three others, *Mya arenaria*, *Schizothaerus capax*, and *Mytilus edulis*, with negative results. Where present, the *Cristispira* seem to be identical with those of *Saxidomus*.

The following is a list of the Lamellibranchiata in the crystalline styles of which *Cristispira* has been recorded, with references to the authorities.

<i>Crassostrea (Ostrea) virginica</i>	Noguchi (10)
<i>Ostrea edulis</i>	Certes (4), Fantham (7)
<i>Crassostrea gigas</i>	Berkeley ( <i>supra</i> )
<i>Saxidomus giganteus</i>	Berkeley ( <i>supra</i> )
<i>Paphia staminea</i>	Berkeley ( <i>supra</i> )
<i>Venus casta</i>	Dobell (6)
<i>Venus mercenaria</i>	Noguchi (10)
<i>Pecten jacobaeus</i>	Gross (8)
<i>Modiola modiolus</i>	Noguchi (10)
<i>Anodonta multabilis</i>	Keysselitz (9), Schellack (11)
<i>Anodonta cygnea</i>	Fantham (7)
<i>Macoma</i> spp.	Rittenberg (unpublished, in litt.)
<i>Tivella stultorum</i>	Rittenberg (unpublished, in litt.)
<i>Siliqua patula</i>	Rittenberg (unpublished, in litt.)
<i>Soletellina acuminata</i>	Dobell (6)

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## ROLE OF GLYCEROL IN THE COLD-HARDENING OF *BRACON CEPHI* (GAHAN)<sup>1</sup>

R. W. SALT<sup>2</sup>

### Abstract

Concentrations of glycerol as great as 5 molal are built up in larvae of *Bracon cephi* (Gahan) after hibernation begins in the fall, and are lost again in the spring. The source of the glycerol was not determined, but is not glycogen. Large solute concentrations, composed mostly of glycerol, depress the melting points of the haemolymph to as low as  $-17.5^{\circ}\text{C}$ . Supercooling points of the intact larvae are lowered even more than their melting points, and this excess is proportional to the melting point depression.

Glycerol is directly responsible for the cold-hardening of the larvae in two separate ways: by increasing supercooling, it increases the ability of the larvae to avoid freezing, and by its protective action it allows the larvae to survive even if they do freeze.

### Introduction

In spite of ingenious precautions no one has yet been able to supercool plain water more than 41 Centigrade degrees ( $^{\circ}\text{C}$ ) and there are good reasons for believing that this figure cannot be much exceeded (7, 14). Solutes depress the supercooling point by lowering the melting point, but the extent of supercooling is probably limited in the presence of solutes as it is in plain water. When I recorded supercooling points as low as  $-47.2^{\circ}\text{C}$  in overwintering larvae of *Bracon cephi* (Gahan), a parasite of the wheat stem sawfly, it seemed, therefore, that their melting points must be very low, or that the solutes in this insect must in some way be able to increase the ability of water to supercool, or possibly both. Tests quickly showed that the melting points of the larvae were extraordinarily low and that supercooling was in fact less than  $40^{\circ}\text{C}$ . Glycerol was found to be the solute mainly responsible for the large melting point depressions, and its effects and possible source are investigated in this paper.

There are few authenticated cases in which an insect is cold-hardened through becoming able to supercool to lower temperatures than it could previously (11). The synthesis and accumulation of low molecular weight solutes thus offers a satisfactory explanation for this type of cold-hardening.

### Materials and Methods

Larvae of the overwintering generation of *Bracon cephi* were collected from wheat stems from the time of cocoon formation in the late summer to the time of prepupal development in the spring.

Melting points of the haemolymph were obtained as previously described (10), except that no Ringer's solution was used. The insect was cut shallowly, the exudate dabbed directly on a cover slip, and quickly covered with another

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slip and sealed. Supercooling points of intact larvae were taken with a 40-gauge copper-constantan thermojunction and electronic recording potentiometer, at a cooling rate of about 1 degree per minute. They were accurate to  $\pm 0.1^\circ\text{C}$ , melting points to  $\pm 0.2^\circ\text{C}$ .

#### *Determination of Glycerol*

Glycerol contents of the larvae were estimated quantitatively by the colorimetric method of Lambert and Neish (4). As this method detects other polyhydric alcohols and also gives a limited reaction with glucose, the glycerol was at first separated on paper and eluted. However, appropriate corrections were necessary because elutions from blank paper gave positive results. When chromatographic tests revealed that no reducing sugars or polyhydric alcohols other than glycerol were present in these larvae, the paper separation was discontinued. The chromatographic method described in a previous paper (12) gave comparable results, but was less suitable than the colorimetric method for quantitative use. Because of the absence of reducing sugars and polyhydric alcohols other than glycerol it is felt that the colorimetric method detected only glycerol, and did not include other substances sensitive to the test such as encountered by Wyatt and Meyer (16).

Larvae were prepared similarly for both methods. Individuals were dried to constant weight in a vacuum over  $\text{CaCl}_2$  at  $50^\circ\text{C}$  for moisture content determinations, and were then ground up singly or in groups in 80% ethanol and No. 400 Carborundum powder. The mixture was centrifuged, the supernatant removed, and the residue washed with more ethanol and centrifuged twice more. Tests revealed that less than 1% of the glycerol then remained in the residue. The three ethanol extractions were combined, dried at  $50^\circ\text{C}$ , and the residue from this taken up in a known volume of water, usually 0.3 ml. These preparations were stored at  $-50^\circ\text{C}$ .

Glycerol concentrations are expressed either as molality or percentage weight, the latter being glycerol as a percentage of moisture content plus glycerol.

To determine whether significant quantities of glycerol were lost by evaporation during the 3 days required to dry the larvae to constant weight in a vacuum, a comparison was made of the glycerol concentrations of such larvae with similar larvae that were not subjected to drying. From two groups of 37 larvae two sets of 31 larvae each were selected such that each set had the same mean supercooling point. This ensured as well as possible that the mean glycerol concentrations of the two sets were approximately equal. Duplicate measurements of glycerol were made on each larva and calculated as percentages of fresh weight. The dried set contained an average of 10.2% glycerol and the fresh, or undried, set 9.8%; hence no appreciable amount of glycerol was lost during the vacuum drying used to determine moisture content.

#### *Determination of Glycogen and Glucose*

As glycogen appeared to be the logical source of glycerol in *B. cephi*, it was measured in both cold-hardy and non-hardy larvae by the method of Kemp

and Kits van Heijningen (3). Glucose was measured in the same material by the method of Mendel, Kemp, and Myers (8), and its absence in cold-hardy larvae was determined chromatographically.

#### *Determination of Fats*

Determination of fats as ether extracts of dried larvae was made only to obtain a rough idea of the comparative amounts in cold-hardy and non-cold-hardy larvae. Dried larvae were ground up in groups of 15 and extracted with four 1-ml volumes of ether. The evaporated extracts were weighed and calculated as percentages of dry matter.

### Results

#### *Seasonal Changes in Cold-hardiness*

When the overwintering brood of larvae spin their cocoons they already possess a fair degree of cold-hardiness, being able to supercool to about  $-30^{\circ}\text{C}$ . However, their exposed hibernation quarters in the stems of wheat, which are likely to protrude above the snow, makes additional cold-hardiness necessary. Table I shows that supercooling points fell during the fall and early winter, and to a considerable extent also at a constant temperature of  $5^{\circ}\text{C}$ . By late November, in 1957, larvae had acquired the ability to avoid freezing during any but the most extreme cold weather experienced in their habitat and territorial range. Cold-hardiness was quickly lost with the advent of warm weather in the spring.

The lowest supercooling and melting points recorded were  $-47.2$  and  $-17.5^{\circ}\text{C}$  respectively, for larvae collected and frozen in November.

TABLE I

Seasonal and temperature-induced changes in supercooling points, melting points, and glycerol concentrations of *B. cephi* larvae (samples range from 20 to 100 larvae)

Collection date	Treatment	Supercooling point, $^{\circ}\text{C}$	Melting point, $^{\circ}\text{C}$	Glycerol concentration, molal
Aug. 15/58	None	$-29.0$	$-1.5$	0.02
Sept. 28/55	None	$-29.6$	—	—
Sept. 28/55	$+5^{\circ}\text{C}$ , 12 wk.	$-37.6$	—	—
Sept. 28/55	Outdoors, 12 wk.	$-40.5$	—	—
Nov. 22/57	$-5^{\circ}\text{C}$ , < 3 wk.	$-41.2$	$-8.8$	2.74
Nov. 22/57	$-5^{\circ}\text{C}$ , 3-4 mo.	$-40.1$	$-6.8$	2.80
Nov. 22/57	$-5^{\circ}\text{C}$ , 8 mo.	$-36.0$	—	2.13
Apr. 1/58	$-5^{\circ}\text{C}$ , 1 wk.	$-37.6$	$-6.7$	2.04
May 2/58	None	—	$-1.4$	0.25
May 2/58	$20^{\circ}\text{C}$ , 1 wk.	$-26.0$	—	0.03

#### *Influence of Melting Points on Supercooling Points*

Seasonal changes in the supercooling points are closely related to changes in the melting points, but, surprisingly, they are somewhat greater than the latter (Table I). This is illustrated more precisely in Fig. 1, where paired values for 79 larvae are plotted. Had the amount of supercooling remained

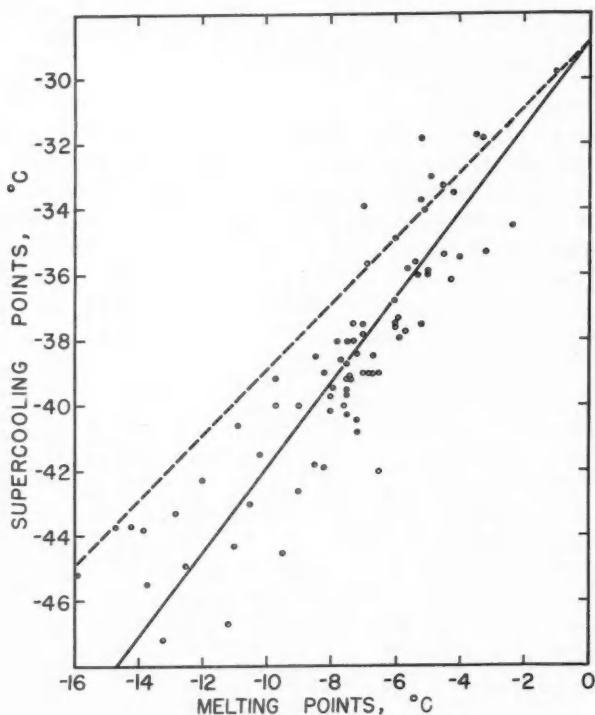


FIG. 1. Relation between supercooling points and melting points of 79 *B. cephi* larvae of variable cold-hardiness. Regression line solid. Broken line indicates slope for theoretical condition of constant amount of supercooling. Divergence indicates increase in supercooling.

constant the regression would follow or parallel the broken line; instead, the calculated regression line (solid) demonstrates that the *extent of supercooling* increased as the melting points decreased.

#### *Influence of Solutes on Melting Points*

##### *(a) Glycerol*

Glycerol was chosen as the solute most likely to be responsible for the very low melting points observed in *B. cephi* larvae. As it was not feasible to measure both glycerol concentration and melting point in the same individual because of *Bracon's* small size (0.8 to 8.0 mg, averaging about 3 mg), the relation of melting points to glycerol concentrations was obtained indirectly from the relation of each of these to the supercooling points. Figure 2 shows the relation between supercooling points and glycerol contents of 109 larvae. Supercooling changed by an average of 3.9 C° for each molal unit of glycerol.

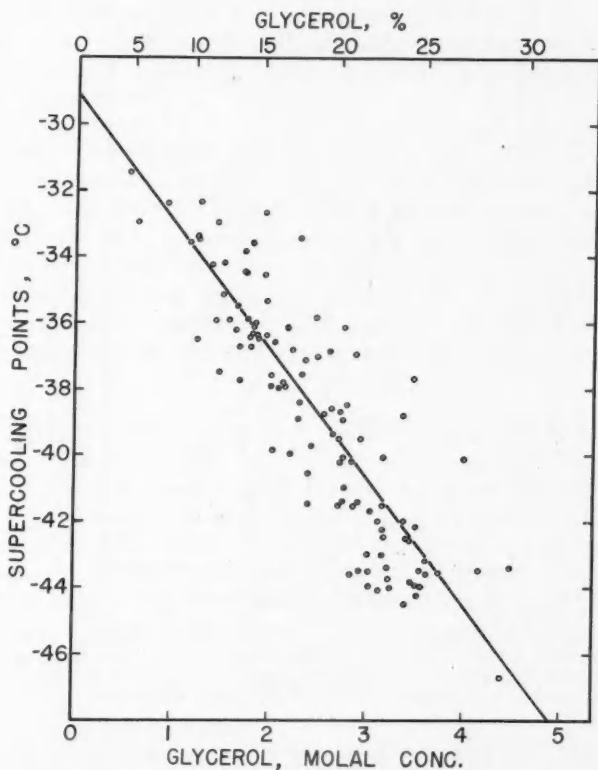


FIG. 2. Relation between supercooling points and glycerol concentrations in 109 *B. cephi* larvae of variable cold-hardiness.

From the regression lines of Figs. 1 and 2, the mean relation between glycerol concentration and melting point was calculated, and is shown by the lower curve in Fig. 3. The upper curve, disregarding for the present the solid dots, is the freezing point curve for pure glycerol-water solutions (15). Though the glycerol must account for most of the melting point depression in the larvae, the discrepancy between the two curves shows that the observed concentrations of glycerol are not sufficiently high to account for all of it. The difference between the slopes of the straight portions of the two curves was significant at the 1% level.

#### (b) Other Solutes

Solutes other than glycerol are of course present. That they are present in usual total amounts is indicated by the melting points of fall larvae that have barely begun to synthesize glycerol, and of spring larvae that have almost totally reconverted it (Table I). As the melting points of glycerol-free larvae are approximately  $-1.5^{\circ}\text{C}$ , the curve has accordingly been bent

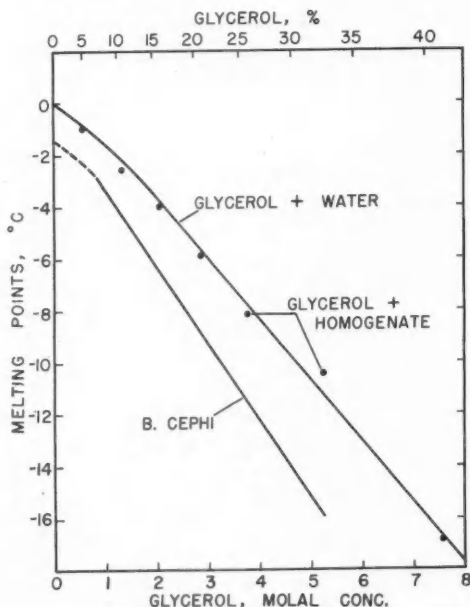


FIG. 3. Lower curve: Relation between melting points and glycerol concentrations in *B. cephi* larvae, calculated from the regression lines of Figs. 1 and 2. Upper curve: Freezing point curve of glycerol and water, from data of Spangler and Davies (15). Solid dots: Melting points of a homogenate of *H. cecropia* pupae with added glycerol.

as shown by the dotted line in Fig. 3. Near this end of the curve the melting-point depression is adequately explained by the sum of the glycerol and other solutes, but as the glycerol concentration approaches 5 molal, it falls short of accounting for the observed melting points by about one molal unit.

Chromatographic tests for polyhydric alcohols other than glycerol, and for reducing sugars, were negative in cold-hardy larvae. Colorimetric methods revealed small amounts of glucose in non-hardy larvae and traces in cold-hardy larvae.

Ash content of cold-hardy larvae after 7 hours at 500° C was less than 2% of the dry-matter content.

### (c) Solvent Changes

If part of the moisture content of the larvae were unavailable as solvent, say as bound water, the effective glycerol and other solute concentrations would be greater than those actually calculated. To test this, known amounts of glycerol were added to a homogenate whose moisture content was first determined from an aliquot. As *B. cephi* larvae are so small, the homogenate was prepared instead from two pupae of *Hyalophora cecropia* (L.) whose natural glycerol content was less than 0.1 molal. The melting points of the mixtures are represented by the solid dots in Fig. 3. These fall so close to

the glycerol-water curve that it is concluded that all of the water in the homogenate acted as solvent for the glycerol. Unless living larvae of *B. cephi* react differently, it appears that little if any water is bound or removed from the role of solvent.

#### *Glycogen and Glucose Content*

The glycogen content of *B. cephi* larvae varied between  $\frac{1}{3}$  and 2% of the total weight, and there was no significant difference between cold-hardy and non-hardy individuals. Thus glycogen is the source of little, if any, of the glycerol found in cold-hardy larvae. Glucose determinations made at the same time ran as high as 2% in non-hardy larvae, but dropped to 0-0.4% in cold-hardy larvae. As already mentioned, chromatograms of the latter larvae were negative for glucose and other reducing sugars.

#### *Fat Content*

With glycogen ruled out as major source of glycerol, fats were next considered. The tests made for fat are acknowledged to be cursory and inadequate, but were made on the chance that gross differences might be exhibited. Non-hardy fall larvae containing no glycerol contained 42% fat as percentage of dry matter. Cold-hardy larvae with a mean glycerol concentration of 2.13 molal contained 35% fat. A set of cold-hardy larvae incubated 1 week at 20° C, in which time the glycerol should have almost disappeared (cf. Table I), contained 38% fat.

### Discussion

#### *Demonstration of Cold-hardening*

In an earlier paper (9) the author expressed doubt that insects were able to develop increased cold-hardiness. At that time it was demonstrated that an insect's tissues possessed a characteristic level of cold-hardiness, and although this level was readily reducible by various factors, such as the presence of fresh food in the alimentary tract, it did not appear capable of much, if any, increase. The regaining of the normal or inherent level when feeding ceased before molting or hibernation was considered not to be cold-hardening in the true sense. A later paper (11) considered the obvious lowering of freezing and supercooling points by solute-concentrating caused by desiccation, but showed that this could produce only minor increases in cold-hardiness unless the insect was severely dehydrated. Conditioning at temperatures near 0° C was without effect on the cold-hardiness of two out of four species studied at that time, but the remaining two species definitely demonstrated that true cold-hardening occurs in some insects. One was *Bracon cephi*, whose average supercooling point dropped 8 C° in 12 weeks at 5° C, and 11 C° during the same period outdoors (11, and Table I). The other was *Loxostege sticticalis*, the beet webworm, which cold-hardened about 4 C° in 12 weeks outdoors, and less at constant temperatures. Both species contain glycerol (12, 13), though in very different concentrations.

When larvae of *Cephus cinctus*, the hosts of *Bracon cephi*, complete the spinning of their cocoons, they possess a level of ability to supercool that will not later increase. At a similar stage the parasite larvae are slightly hardier than their hosts, but this level is a minimum (Table I). In response to the decreasing temperatures of fall the synthesis of glycerol begins and cold-hardiness increases accordingly. In spring the process is reversed.

It is seen, then, that some species of insects truly cold-harden by synthesizing low molecular weight solutes, which lower both melting and supercooling points. In the case of glycerol and some similar neutral solutes, a second type of cold-hardening also occurs, as such solutes protect tissues from injuries otherwise caused by freezing. Cold-hardy larvae of *B. cephi* are not likely to freeze, having very low supercooling points, but if they do freeze they survive, owing to the protective action of glycerol. In fall and spring, without glycerol, larvae are killed by freezing.

#### *Influence of Solutes on Cold-hardening*

The concentrations of glycerol measured in cold-hardy *B. cephi* larvae leave no doubt that glycerol is the major cause of their phenomenal cold-hardiness. The surprising thing is that the observed glycerol, added to the solutes present in the non-hardy larvae, fails to account for all of the observed melting point depression (Fig. 3). The cause of the extra depression is not known, but it is not due to other polyhydric alcohols, reducing sugars, or bound water. Nor is it the result of a high salt content, for less than 2% of the dry matter was ash. Even if all this were NaCl it would only form a 0.3 molal solution with a melting point depression of about 1 C°.

Unless glycerol was underestimated by about 15–20%, other solutes must be responsible for the unexplained portion of the melting point depression. Whatever these solutes may be, they are present in quantities proportional to the glycerol.

The possibility that the haemolymph contains more glycerol than the remainder of the body, suggested by the fact that its melting point is much lower than is attributable to the glycerol measured in the whole insect, is not likely. If there were considerable differences in the glycerol content of the various tissues, then that tissue with the least glycerol would possess the highest melting point, would supercool least, and would consequently determine the supercooling point of the whole insect. The remaining tissues would then possess a greater potential for supercooling than is actually recorded, but as the latter amount is already very great, it is hardly likely to represent a minimum. Moreover, the close dependence of supercooling on the glycerol concentration of the whole insect, shown in Fig. 2, suggests that no tissue has appreciably less glycerol than any other.

#### *Influence of Solutes on Survival After Freezing*

The probable presence of a large non-glycerol solute content raises an interesting question regarding the nature of freezing injury and the action of

glycerol and other neutral solutes in protecting against such injury. Lovelock (5) pointed out that the presence of neutral, non-toxic solutes ensures that electrolytes will reach toxic concentrations only at lower temperatures than otherwise. The amount of protection is proportional to the relative concentrations of neutral solutes and electrolytes. One important assumption is made: that injury is caused by the concentrating of electrolytes. The concentration of these in the unfrozen cell is of course innocuous, but with the removal of water as ice they reach toxic and finally lethal values. When a neutral solute is added it also contributes to the melting point depression, and hence the temperature must be lowered more than before to reach the same lethal electrolyte concentration. In other words, at the temperature at which electrolytes become lethal when present alone they are still sublethal in the presence of neutral solutes. Furthermore, it is the proportion, or ratio, between the substances that will become toxic and those that will not that determines the lethal temperature.

Figure 3 shows that the more concentrated the glycerol in *B. cephi* larvae the greater is the melting point depression attributable to other solutes. This suggests that the hardier larvae may contain a greater concentration of electrolytes than the less-hardy larvae and that more glycerol is thereby required to counteract them. However, as the ash content of cold-hardy larvae indicated that the mineral salts were not especially abundant, it is probable that the electrolytes are low-molecular-weight organic compounds such as amino and fatty acids. Concentrations of these with an accumulative value of up to one mole would account for the unexplained melting point fraction.

#### *Source of Glycerol in B. cephi Larvae*

Chino (1) showed that glycogen was quantitatively converted into glycerol and sorbitol in silkworm eggs shortly after they entered diapause, and was resynthesized from them when diapause ended. Wyatt and Meyer (16) postulated the same source for the glycerol in *Hyalophora cecropia* pupae. However, the low glycogen content of the larvae of *B. cephi*, less than 2%, and the absence of any decrease as glycerol increased, proves that glycogen is not the source of glycerol in this insect. Some of the glycerol could come from fat, but the observed drop in fats as the larvae go into hibernation is hardly sufficient evidence to support a claim that fats are the source of glycerol. Nevertheless the possibility deserves more study.

#### *Increase in Supercooling Caused by Solutes*

Lusena (6) found that neither sodium chloride nor glycerol in concentrations up to 2.5 M changed the ability of water to supercool. In order to counteract the variable nucleating activity of stray motes, he added silver iodide, the most efficient nucleating agent known, and thereby made supercooling more uniform. However, it amounted only to 1.02–2.36 C° in his experiments. In *B. cephi* larvae, on the other hand, supercooling ranged from 26–36 C°, the amount varying in direct proportion to the solute concentration (Figs. 1, 2).

The reason for this relation is not clear. Increasing viscosity of the glycerol does not seem to be the cause, as it had no noticeable effect in Lusena's solutions. In the larvae, however, lipids, proteins, and other substances would all add to the increasing viscosity as the temperature fell; hence this property could conceivably be responsible for the observed excess of supercooling.

#### *Relation of Glycerol to Diapause in B. cephi*

Chino (1, 2) found that in silkworm eggs glycogen is broken down into glycerol and sorbitol with the onset of diapause, and when diapause is ended the glycogen almost regains its prediapause level. He pointed out that the restoration of glycogen is associated with the diapause break itself, rather than with subsequent postdiapause development, because eggs regained their glycogen content even when embryogenesis was suppressed at 5° C. The situation in *B. cephi* is somewhat similar, even though glycogen is not the source of glycerol in this species. When the diapausing larvae of the overwintering brood spin their cocoons in the fall they contain virtually no glycerol. It may be several weeks before glycerol synthesis becomes appreciable, as a result of the stimulus, probably, of cold nights and warm days. In the spring glycerol is lost rapidly but postdiapause development is concurrent. Larvae stored at -5° C lost no glycerol in the first 4 months, but did so in the next 4 (Table I). As diapause-breaking is very slow at this temperature, it is quite possible that glycerol was maintained during diapause and slowly lost after diapause was broken, as in silkworm eggs.

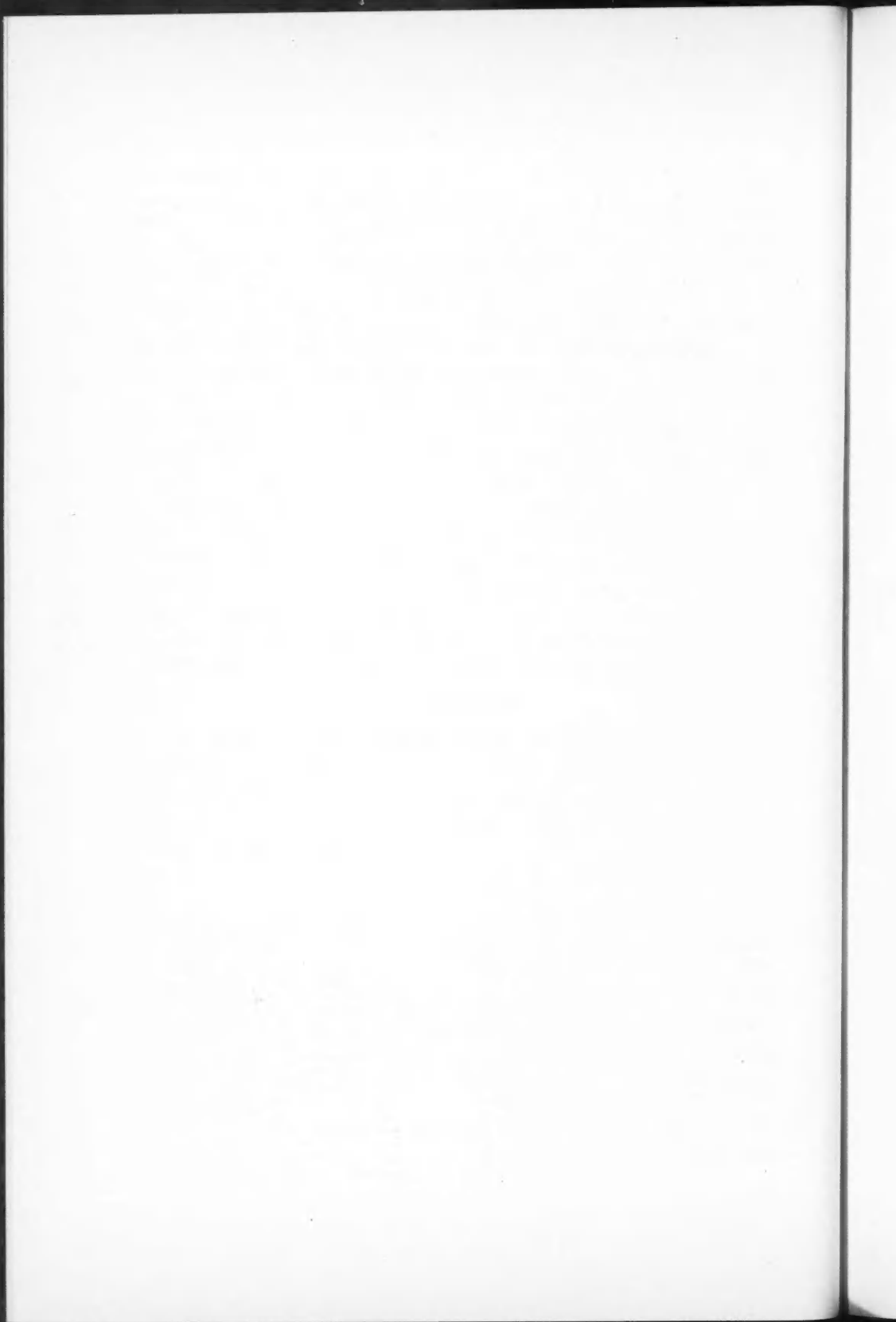
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It is a pleasure to acknowledge the constant interest and advice of Drs. R. Kasting, A. J. McGinnis, and D. W. A. Roberts of this laboratory, and to thank them, as well as Dr. G. R. Wyatt of the Department of Biochemistry, Yale University, Dr. C. V. Lusena of the Division of Applied Biology, National Research Council, Ottawa, and Mr. N. S. Church of this laboratory, for valuable criticisms of the manuscript.

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**NEMATODES FROM VARANID LIZARDS OF BORNEO.  
A DESCRIPTION OF ABBREVIATA BORNEENSIS N. SP.  
(NEMATODA : PHYSALOPTERIDAE) AND RECORDS OF  
TWO OTHER SPECIES<sup>1</sup>**

G. A. SCHAD

**Abstract**

Three nematodes are recorded from varanid lizards of Borneo. The nematodes are *Amplicaecum varani*, *Tanqua tiara*, and *Abbreviata borneensis* n. sp.

Three species of nematodes, one of them new, are herein reported from lizards of the genus *Varanus*. The parasites were collected during the recent expeditions to Borneo by Drs. D. D. Davis and R. F. Inger of the Chicago Natural History Museum. The author wishes to thank Dr. Inger for making this material available for study. Unless otherwise stated, all material is deposited at the aforementioned museum (hereafter abbreviated C.N.H.M.). The parasites comprising this collection are as follows:

- (1) *Amplicaecum varani* Baylis and Daubney, 1922 from the stomach of *Varanus rudicollis* (C.N.H.M. 76292) at Matang, Sarawak, First Division.
- (2) *Tanqua tiara* (von Linstow, 1879) from *Varanus dumerili* (C.N.H.M. 63620) at Dewhurst Bay, Kinabatangan District, North Borneo.
- (3) *Abbreviata borneensis* n. sp. (Figs. 1-7; Table I).

*Diagnosis: Abbreviata borneensis*

Denticulated ridge incomplete, present only in angles of mouth. Four uteri arising dichotomously from common trunk which connects with egg chamber. Left spicule measures 1.94-2.40 mm; grooved throughout most of distal third, becoming solid near tip. Tip usually reflexed to form hook. Right spicule short, slightly bent, with distal, blade-like edge.

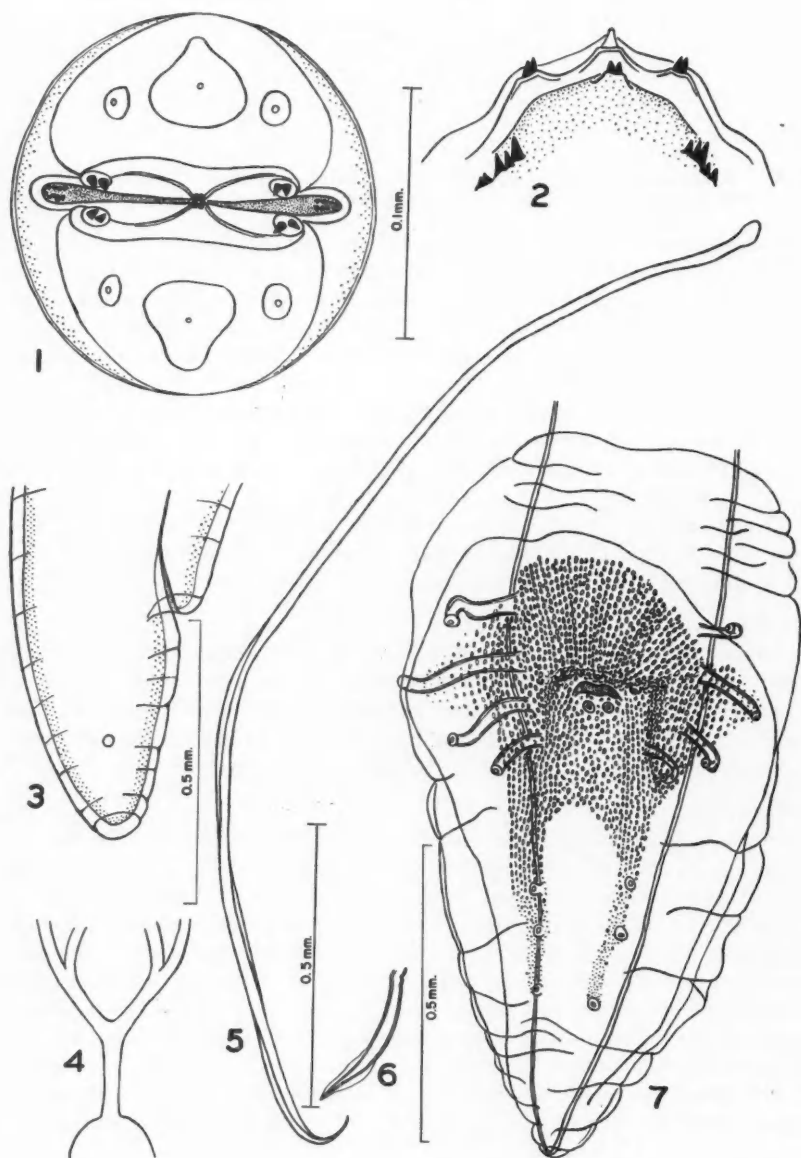
*Description*

Measurements are given in Table I. The mouth is bordered by two lips (Figs. 1, 2), each of which bears a large external apical tooth (the externo-lateral tooth), closely internal to which is situated a smaller bifid internal tooth (the internolateral tooth). From its apex the edge of each lip slopes gradually to the level of the two pairs of submedian teeth where the slope increases markedly toward the angle of the mouth (Fig. 2). In these angles is situated a denticulated ridge. Four circumoral papillae and a pair of amphids are present. The lips are seated in a cuticular depression, which is surrounded by a prominent collar.

The mouth is shallow and is bordered posteriorly by a cuticular thickening capping the anterior end of the oesophagus. The latter consists of a short

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FIGS. 1-7. *Abbreviata borneensis*. FIG. 1. En face. FIG. 2. Inner surface of lip. FIG. 3. Female tail. FIG. 4. Diagram showing the dichotomous origin of the uteri. FIG. 5. Left spicule. FIG. 6. Right spicule. FIG. 7. Male tail.

TABLE I  
Measurements (mm) of *Abbreviata borneensis* n. sp.

	Males	Females
No. specimens measured	14	6
Total length	11.00-24.50	24.00-37.00
Nerve ring from anterior	0.24- 0.37	0.33- 0.42
Cervical papillae from anterior	0.34- 0.53	0.54- 0.68
Excretory pore from anterior	0.37- 0.60	0.62- 0.76
Length of oesophagus (total)	2.40- 3.43	3.19- 4.91
Length of oesophagus (muscular)	0.22- 0.38	0.30- 0.47
Length of left spicule	1.94- 2.40	-
Length of right spicule	0.29- 0.94	-
Vulva from anterior end	-	5.83- 9.26
Vulva ratio	-	1:2.5-1:3.7
Length of tail	-	0.19- 0.50
Egg length	-	0.036- 0.054
Egg width	-	0.021- 0.029

muscular section followed by a long glandular section. The nerve ring surrounds the oesophagus at the posterior end of the muscular section. The small, spike-like cervical papillae and the excretory pore follow in sequence a short distance posterior to the nerve ring.

The vulva, located anterior to the mid-body, is connected to an egg chamber by the vagina. A common uterine trunk leads from the egg chamber to the four uteri which arise dichotomously (Fig. 4). The eggs are small, thick-walled and variable in size. The female tail is rounded at its tip (Fig. 3).

The expanded cuticle of the male tail forms caudal alae (Fig. 7). Four pairs of pedunculated papillae are present; of these the two anterior pairs are preanal while the two posterior pairs are postanal. Five small sessile perianal papillae are situated close to the anal opening with three anterior and two posterior to the anus. Six additional sessile papillae occur in three pairs which are evenly spaced and extend posteriorly from the mid-tail level to a point approximately three-quarters the length of the tail. The perianal field of tubercles is prolonged into two horns which extend posteriorly to the last pair of caudal papillae. The tubercles are arranged in longitudinal rows except at the ends of the horns and in the postanal field. In these areas their arrangement is random. The spicules differ markedly in size and shape. The longer left spicule (Fig. 5) is twisted and grooved throughout most of the distal third of its length. Close to the distal end the groove merges into a solid recurved tip. The shorter right spicule is slightly curved and is characterized by a clear expansion or blade surrounding its tip (Fig. 6).

Host:	<i>Varanus rudicollis</i> (C.N.H.M. 76292).
Location:	Stomach.
Locality:	Matang, First Division, Sarawak.
Type specimens:	Holotype, a male (C.N.H.M.).
	Allotype, a female (C.N.H.M.).
	Paratypes, both sexes (C.N.H.M.).

*Additional material:*

1. Stomach, *Varanus rudicollis* (C.N.H.M. 63622), Sapagaya Forest Reserve, Borneo.
2. Stomach, *V. rudicollis* (C.N.H.M. 76291), Kalabakan, Tawau District, North Borneo (in author's collection).
3. Stomach, *V. salvator* (C.N.H.M. 71580), Santubong, First Division, Sarawak.

*Discussion*

*Abbreviata borneensis* closely resembles *A. oligopapillata* (Kreis, 1940) and *A. bancrofti* (Irwin-Smith, 1922). It differs from the description of the former in the following particulars.

1. According to Kreis (2) the right spicule of *A. oligopapillata* is very characteristic in that its proximal end is very obliquely truncated and the distal third is flexed at almost a right angle. In *A. borneensis* the right, or shorter, spicule is neither sharply flexed distally nor very obliquely truncated proximally. It should also be noted that it has a blade-like, distal expansion which is not mentioned in the description of *A. oligopapillata*.

2. The left, or longer, spicule of *A. borneensis* has a trough-like groove throughout its distal third to close to the tip where it comes to an end as it merges into a solid sharp point. The latter is usually recurved to form a "fish-hook", but occasionally is secondarily bent so as to be S-shaped. The left spicule of *A. oligopapillata* is described merely as being narrow and ending in a fine point.

3. In *A. oligopapillata* the posterior extensions of the field of tubercles on the tail of the male reach the first or anterior pair of sessile papillae, whereas in *A. borneensis* the extensions of the tubercular field reach the third or posterior pair of papillae.

4. Kreis states that papillae are lacking along the posterior border of the anus in *A. oligopapillata*. These are present in *A. borneensis*.

Differentiation of *A. borneensis* from *A. bancrofti* as described by Irwin-Smith (1) is based on the following:

1. A tooth, present below each pair of submedian teeth in *A. bancrofti*, is absent in *A. borneensis*.

2. In *A. borneensis* the field of tubercles on the male tail extends posteriorly along the paired sessile papillae in two separate prolongations, whereas in *A. bancrofti* the field of tubercles is not separated into two prolongations but extends posteriorly as one continuous field.

3. The left, or longer, spicule is not recurved in *A. bancrofti* as it is in *A. borneensis*.

4. The pericloacal rim said to be present in *A. bancrofti* is absent in *A. borneensis*.

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## STUDIES ON STRONGYLOIDES OF PRIMATES

### V. SYNONYMY OF THE SPECIES IN MONKEYS AND APES<sup>1</sup>

PREMVATI<sup>2</sup>

#### Abstract

A study of the comparative morphology and life history of *S. fülleborni*, *S. cebus*, and *S. simiae* in both the parasitic and free-living generations under different environmental conditions, and their comparison with the free-living stages from faeces of Old World and New World primates has led to the conclusion that the three species should be synonymized into one, for which the name *Strongyloides fülleborni* von Linstow (1905) has priority.

Three species of *Strongyloides* have been described from apes and monkeys: *S. fülleborni* von Linstow (1905) from *Anthropopithecus troglodytes* and *Cyanopcephalus babuin*, *S. cebus* Darling (1911) from *Cebus hypoleucus*, and *S. simiae* Hung and Hoeppli (1923) from *Macaque* sp. *S. fülleborni* has been re-described by Sandground (12), Goodey (3), and Premvati (8); *S. cebus* by Sandground; and *S. simiae* by Chandler (1) and Kreis (5).

My study of the morphology and biology of *S. fülleborni* from rhesus monkeys, the examination of free-living stages of *Strongyloides* from fresh faeces of the chimpanzee, baboon, green African monkey, rhesus monkey, squirrel monkey, spider monkey, and sapajou monkey from the Zoological Gardens, Granby, Quebec, and the available literature, have led me to re-examine the characters used to distinguish these three species.

The only characters by which the parasitic female of any species of *Strongyloides* can be distinguished are: size, ratio of the oesophagus to body length, the shape and size of the tail, the form of cuticle, position of the vulva, disposition of the ovaries, size and number of eggs in the uterus, and the stage of the parasite in fresh faeces. The characters by which the free-living generation can be distinguished are: size of the mature female, oesophagus of the female, shape and size of the vulva, size and number of eggs in the uterus, size of the mature male, size of spicules and gubernaculum, number of preanal and postanal papillae, size of infective larvae, and ratio of oesophagus to body length of infective larvae. I have compared the measurements of these three species given by various authors (1, 2, 3, 4, 5, 6, 12) and compared them with measurements made by myself. In all cases, except that of the size of the parasitic female of *S. fülleborni* given by Sandground (12) and Goodey (3), these measurements fall within the range of normal variation and cannot be used to distinguish species. In the case of *S. fülleborni*, Sandground

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gave the length as 2.02 to 2.85 and Goodey as 2.03 to 2.96 mm, whereas von Linstow (6) gave it as 3.78 mm, and I found it to be from 3.6 to 4.6 mm in my specimens.

The lack of well-defined characters by which species of this genus can be recognized has led many workers to erect a new species based only on a new host record, or on its differences from *S. stercoralis*. Many new species have been described mainly on body dimensions and the descriptions rarely contain adequate illustrations. While in creating a new species, some workers have considered only the parasitic generations, others have used only the free-living females.

Hung and Hoeppli considered that the relation between the length of the oesophagus and total body length is a distinctive character and they used it as one of the major distinctions between *S. fülleborni* and *S. simiae*. In 10 specimens of *S. simiae* they found the oesophagus to be between  $\frac{1}{4}$  and  $\frac{1}{3}$  of the body length. Chandler and Kreis found this same relationship in their specimens. In *S. fülleborni*, von Linstow found the oesophagus to be  $\frac{1}{4}$ , Sandground  $1/3.6$  to  $1/4.3$ , Goodey between  $\frac{1}{4}$  and  $\frac{1}{3}$ , and I  $\frac{1}{3}$  to  $\frac{1}{4}$  of the body length. Darling made this ratio in *S. cebus* as  $\frac{1}{3}$  to  $\frac{1}{4}$ . Thus, in all three species the ratio is almost the same.

Sandground discussed and illustrated the variations found in the shape of the tail and concluded that this character could not be used for specific purposes. Chandler, however, in dividing the genus into two groups stated: "the 'papillosus' group has a body sharply constricted immediately behind the anus and ending in a short finger-like tail bluntly rounded at the tip, while in the 'stercoralis' group the body tapers evenly from a point some distance in front of the anus and ends in an evenly tapering conical tail, bluntly pointed at the tip." According to Chandler, *S. fülleborni*, *S. cebus*, and *S. simiae* belonged to the 'papillosus' group and so have the same type of tail. I have always found the shape of the tail in my specimens to be finger-like, bluntly rounded at the tip, and with a sharp constriction immediately behind the anus. In the diagrams of von Linstow and Goodey of *S. fülleborni*, and of Kreis of *S. simiae* the same-shaped tail is shown. The tail of *S. cebus* has not been clearly illustrated in any paper.

Von Linstow and Darling have described *S. fülleborni* and *S. cebus* as having a smooth cuticle; Hung and Hoeppli have described *S. simiae* as having a striated cuticle, which has been regarded as another specific difference. Subsequently, Chandler expressed doubt of this character, noting that it would be very unusual, if not unique among nematodes, if closely related members of a single genus were found to differ in the presence or absence of cuticular striations. Sandground and Goodey observed that all their specimens of *S. fülleborni* were finely striated and that as the striations could be seen only under very high magnification, the earlier authors must have overlooked them. I agree with Sandground and Goodey, having found cuticular striations in all my specimens; these striations can be seen easily by using the gum-arabic - cresyl-blue stain method.

All authors agree that the vulva lies at the junction of the middle and posterior third of the body.

In certain species of *Strongyloides*, the disposition of the ovaries is of diagnostic significance. All authors, including myself, find that both ovaries of all monkey species are sinuous or twisted but that the posterior one has fewer loops than the anterior.

The size of *Strongyloides* eggs is given by various authors as follows: von Linstow, 0.052 by 0.031 mm for *S. fülleborni*; Darling, 0.064 by 0.040 mm for *S. cebus*; Hung and Hoeppli, 0.05 to 0.055 mm by 0.03 to 0.036 mm for *S. simiae*. I have found that the size of the eggs varies with the developmental stage of the embryo present, the length being from 0.049 to 0.063 mm, the breadth from 0.026 to 0.039 mm.

Von Linstow gave the number of eggs in the uterus of *S. fülleborni* as 30, Darling of *S. cebus* as 6 to 8, and Kreis of *S. simiae* as 5 to 20. I have found (10) that, although never more than 8 to 10 eggs are seen in the uterus of the parasitic female at any one time, it is always possible to find, in the intestinal contents of the monkey, strings of as many as 15 to 30 eggs all in the same stage of development. If these eggs had passed out of the uterus at intervals, some at least in the same string would have been at different stages of development.

In *S. stercoralis*, larvae have already hatched from the eggs when they are passed in the faeces. In *S. fülleborni* and *S. cebus* the larvae are still contained within the eggs when they appear in fresh faeces. Chandler noted that in *S. simiae* only eggs containing developed or partially developed embryos are present in fresh faeces. In all three species the egg does not hatch until it is outside of the body.

### The Free-living Generation

*S. fülleborni*, *S. cebus*, and *S. simiae*, under optimum conditions, all show predominantly indirect or heterogonic development (9), that is, both males and females develop from eggs of parasitic females and give rise to larvae which metamorphose into infective larvae. Rhabditiform larvae, whether developed from eggs of parasitic females or from free-living females are in a continuous state of growth and molting and consequently are difficult to compare. But the adult stages or the infective larvae could show constant structures. Looss (7) stated that: "The free living generations, on the contrary, so far as I am acquainted with them, show slight but distinct differences from one another, which make it probable that different species exist." Goodey agreed with Looss.

Sandground observed that the size of the individual of the free-living generation varies considerably with the temperature; its maximum size is attained at the optimum temperature, a reduction taking place at lower temperatures. Chandler was also of the opinion that there are great variations in the free-living generation due to external conditions. I (11) have also observed that not only does the size differ but morphological changes

occur due to temperature. Some of the free-living females developed from the faeces of the chimpanzee and green African monkey have a length of up to 1.30 mm, while the width at the vulva is 0.091 mm and behind the vulva 0.075 mm. There are no doubt slight variations in the sizes of free-living females of all three species but as these variations occur within a species in the same host, they cannot form the basis on which to designate the species.

Von Linstow found the ratio of the oesophagus to total body length of *S. fülleborni* to be  $\frac{1}{6}$ , while Goodey observed it to be  $\frac{1}{3}$  to  $\frac{1}{6}$ . In *S. cebus* Darling observed the ratio to be about  $\frac{1}{3}$ . In *S. simiae*, according to Hung and Hoeppli and to Chandler it is  $\frac{1}{3}$  to  $\frac{1}{6}$ , and according to Kreis,  $\frac{1}{6}$  to  $\frac{1}{3}$ . In *S. fülleborni* I found that the oesophagus varies from  $\frac{1}{6}$  to  $\frac{1}{3}$  of the total body length. In addition to the variation in the proportionate size of the oesophagus, the length changes with the temperature. At minimum developmental temperatures, the total body length decreases with the increasing length of the oesophagus.

The most conspicuous difference observed in females of these three species from monkeys and apes is associated with the vulva. In *S. fülleborni* both von Linstow and Goodey noted that the lips of the vulva are very conspicuous and that the body anterior to it is considerably wider than posterior to it. In describing *S. cebus*, Darling stated: "The vulva of the females is not nearly so prominent as the one figured by von Linstow for *S. fülleborni*, and there is no diminution of the caliber of the female just posterior to the vulva." Sandground noted that: "This is a constant difference between the two species and is the most prominent differential character." Hung and Hoeppli were of the opinion that in *S. simiae* the free-living females did not have a prominent vulva, but Chandler stated that in *S. simiae*: "The vulva is very prominent, the diameter through this point being from 4 to 7  $\mu$  greater than the maximum diameter elsewhere. The body is slightly constricted immediately behind the vulva and then widens out again, but is never quite as wide behind the vulva as before it." Kreis did not mention this in his description of *S. simiae* although in his diagram of the female, he has shown a vulva with salient lips and no constriction behind the vulva.

My specimens of free-living females of *S. fülleborni* from rhesus monkeys and from faeces of both New World and Old World monkeys all showed a prominent vulva with a marked constriction just behind it. This prominence is very great in specimens developed from the faeces of the chimpanzee, baboon, and green African monkeys; it is slightly reduced in those from rhesus, squirrel, spider, and sapajou monkeys. This appearance is perhaps due to the total width of the female attained in a particular host, as the difference in the width of the female before and after the vulva is practically the same in all cases. It is difficult to believe that all these primates are infected with different species of *Strongyloides*. The slight changes in the morphology of the free-living adult females could be due to host variations. The only difference found in the free-living females of New World monkeys

is that they are slightly thinner and smaller in length than those from Old World primates; otherwise, their general appearance is very similar when they are developed at the optimum temperature.

Furthermore, I (11) have noted that the shape and size of the vulva changes with the change in temperature at which development takes place. Females developed at the optimum temperature have very prominent vulvas with a marked constriction which gives a waist-like appearance. In all respects they resemble the females of *S. fülleborni*. At suboptimum temperatures the females lose their waist-like appearance and do not have so prominent a vulva; they resemble those of *S. cebus*.

The shape and size of the vulva of free-living females are also found to change with age. Those of *S. fülleborni* lose their characteristic vulva and waist-like appearance as they become old and unable to lay eggs. The prominence of the vulva of the free-living females is associated with the number of embryonated eggs present in the uterus.

The number of eggs in the uterus of the free-living female has also been used as a specific character. Von Linstow found 8 in *S. fülleborni*, Chandler 15 to 27, and Kreis 4 to 16 in *S. simiae*. The number of eggs present in the uterus of *S. cebus* is not mentioned. The females developed at the optimum temperature from the cultures of rhesus monkeys show from 18 to 36 eggs before the first laying, 15 to 25 before the second or third laying, and so on; the number is reduced as the female becomes old. Moreover, at temperatures lower or higher than the optimum, the number of eggs is always less than at the optimum temperature. This factor may depend on the possibility of the females mating, an act which is reduced at both lower and higher temperatures.

The developmental stage of the ova of the free-living female is also slightly variable at different temperatures. At the optimum temperature, after 48 hours' incubation, all the eggs in the uterus are embryonated. These eggs, when laid, take no more than half-an-hour to hatch into rhabditiform larvae. On the other hand, the females developed at lower temperatures mature more slowly.

The size of the males ranges from 0.80 to 0.95 mm and depends on the condition of nourishment, temperature, consistency of the culture, oxygen, and pH of the culture in which they develop (9). The optimum conditions give rise to healthy males of maximum size, while under unfavorable conditions, the males remain thin and small in size.

The size of the spicules and gubernaculum is practically the same in all three species. The shape of the gubernaculum in the three species has been described as flattened and broad in the middle with finger-like tips at its two ends.

The caudal papillae have been described as two pairs—one preanal and one postanal—from all three species. *S. fülleborni* has one very distinct pair of preanal and one pair of postanal papillae, but there are also three additional very small pairs which could be seen only under the highest magnifica-

tion. There are, therefore, five pairs in all—two preanal and three postanal. Due probably to their extremely small size these were not noted by earlier workers. In free-living males developed from faeces of all other primates which I examined, it was possible to count five pairs.

The infective larvae developed from the faeces of the various primates examined by me range in length from 0.40 to 0.75 mm and figures given by different authors all fall within this range.

My investigation confirms Sandground's opinion that the size of infective larvae varies with the age, and with the temperature and nourishment to which larvae are subjected during development.

The ratio of oesophagus to total body length of infective larvae is not given for either *S. fülleborni* or *S. cebus*. Chandler gave 2.1 to 2.3 and Kreis 2.9 to 5.0 for *S. simiae*. The ratio in infective larvae of *S. fülleborni* from rhesus monkeys varies from 2.1 to 2.4 but in larvae developed from the faeces of the other primates it varies from 2.0 to 3.0.

### Summary and Conclusions

In discussing the species of *Strongyloides* Sandground considered that there is no clear case for distinguishing *S. simiae* from the other two species described from primates. He was of the opinion that *S. cebus* is a distinct species from *S. fülleborni*. Chandler considered the morphology and biology of the parasitic parthenogenetic females to be more reliable in distinguishing the various species than is the morphology of the free-living generation which shows more variation. On the other hand, both Looss and Goodey considered the free-living generation to have more distinct characters.

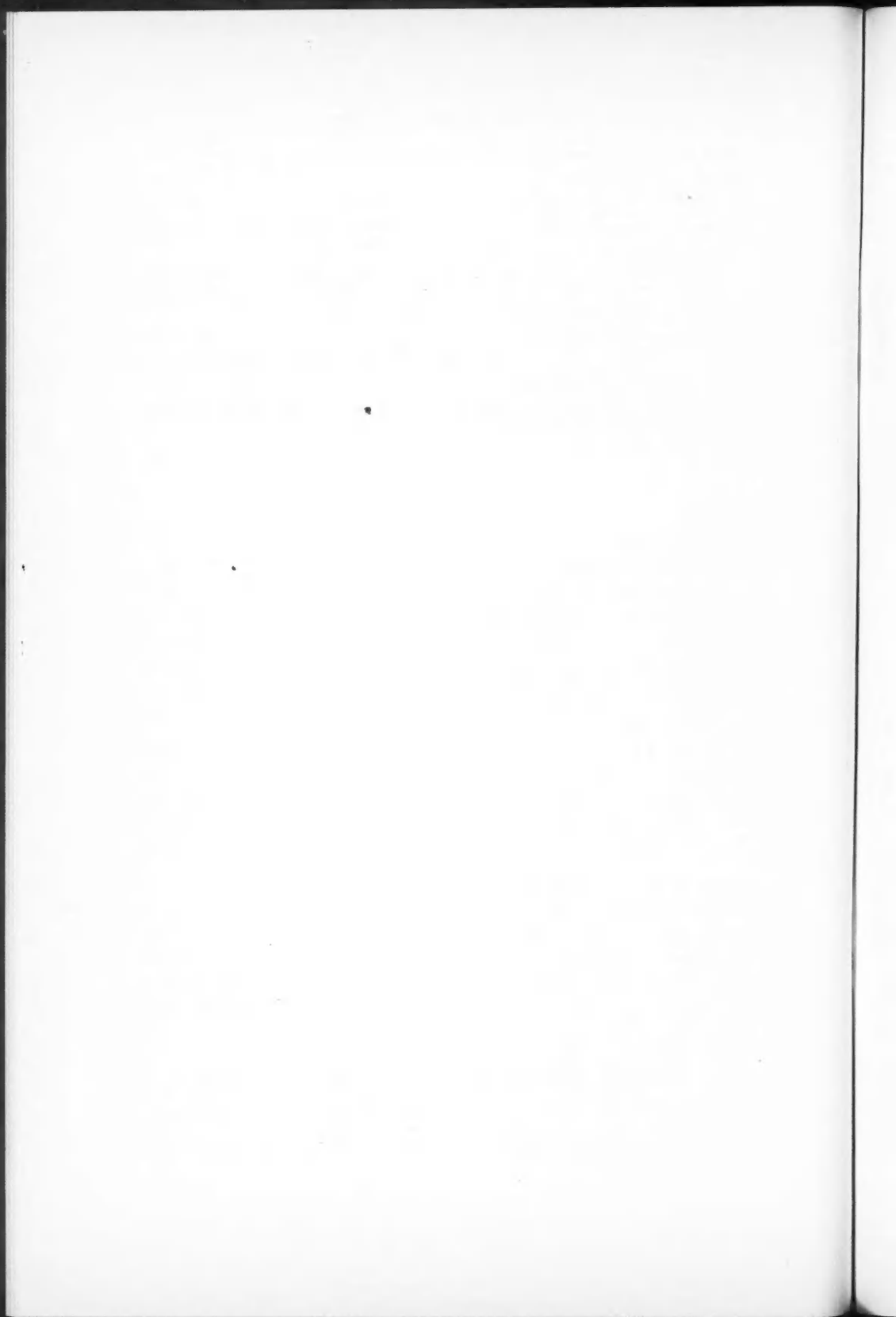
My studies on the morphology and biology of the free-living stages of *S. fülleborni* from rhesus monkeys have shown that free-living females show great variations under different conditions. In addition to variations in size, the vulva changes considerably with changes in temperatures; its shape is affected by age and the number of embryonated eggs in the uterus. Under such circumstances the vulva of the adult free-living females fails as the basis for distinguishing species.

In view of the comparative morphology and life history of *S. fülleborni*, *S. cebus*, and *S. simiae* in both the parasitic and free-living generations under different environmental conditions and their comparison with the free-living stages from faeces of Old World and New World primates, I conclude that the three species should be synonymized into one, for which the name *Strongyloides fülleborni* von Linstow (1905) has priority.

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NOTES

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**ECHINOCOCCUS GRANULOSUS IN DOMESTIC ANIMALS  
IN WESTERN PAKISTAN**

GEORGE LUBINSKY

During the course of a protozoological and helminthological study 14 visits were made between June and December 1953 to an abattoir in Rawalpindi to collect material. Hydatid cysts were found in five (2.1%) of the carcasses of 237 Jamnapari goats, in three (4.6%) of 65 sheep, in four (15.4%) of 26 cattle, and in the 1 water buffalo examined. (More than two full buckets of cysts up to 4 in. in diameter were removed from the lungs and liver of the buffalo.) Most of the cysts were of the unilocular type although multilocular cysts were found in two cows examined.

Adult *Echinococcus granulosus* were found in the intestines of 2 out of 11 stray dogs shot in Rawalpindi during 1953.

Most of the abattoirs in Pakistan—particularly those in rural areas—dispose of offal (including lungs and livers infected with hydatid cysts) by dumping it outside the abattoir. Slaughtering normally takes place during the evening and night when scavenger birds are inactive, and the offal is consumed mostly by stray dogs, jackals, hyenas, etc. These animals serve as a source of infection both to wildlife and to the human population in whom hydatid cysts are not uncommon.

Control of hydatids in Pakistan could easily be effected by either proper disposal of offal or by barring access to it to dogs and wild carnivores.

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**UNSUCCESSFUL ATTEMPT TO DETECT THE PRESENCE OF A TOXIC  
FACTOR IN THE BLOOD OF IRRADIATED RATS**

JANE M. MANSON AND J. A. R. CLOUTIER

A number of studies on circulating humoral factors in the blood of irradiated animals have been reported in the literature. The contradictory results of these studies were reviewed by Lawrence *et al.*, 1948 (3). A transient reduction in white blood cell count of non-irradiated animals after injection of serum from donor animals irradiated a short time before bleeding was reported by Rode (1950(4)). More recently Edelmann (1957(2)) has confirmed the existence of a toxic factor in the blood of rats that had been exposed

to X radiation. The experimental plan was to inject the serum of rats or mice that had been subjected to X rays into unirradiated rats or mice and to observe the effects. Some of the recipients were sensitized by removal of their adrenal glands. The death rate of the injected rats was reported to increase significantly.

TABLE I  
Treatments

Group	Donor	Recipient
1	Unirradiated	Unirradiated
2	Unirradiated	Sensitized with 525 r (Co <sup>60</sup> gamma rays)
3	Irradiated at 900 r (Co <sup>60</sup> gamma rays)	Unirradiated
4	Irradiated at 900 r (Co <sup>60</sup> gamma rays)	Sensitized with 525 r (Co <sup>60</sup> gamma rays)

Early this year, experiments were performed in this laboratory to observe the reported effect with a view to studying, eventually, the origin of the toxic factor. No positive results were obtained. However, Campo *et al.* (1958(1)) have recently reported a failure to demonstrate toxic factors in the serum of irradiated rats. Since our investigation involved a different strain of animals and a different age group, it is thought that these results would be of interest to supplement the findings of Campo *et al.*

Male rats of Wistar strain, 8 to 10 weeks old, served as donors and receivers and were arranged in donor-receiver pairs according to litter, body weight, and age. The rats were placed in four treatment groups of five pairs each, as shown in Table I. The irradiation was performed with the Co<sup>60</sup> teletherapy unit of the Physics Section, Commercial Products Division, Atomic Energy of Canada Ltd. The serum was obtained by anesthetizing the rats with ether until respiratory arrest, severing the carotid arteries, collecting the blood, allowing it to clot at 5-8° C for 12 minutes, and centrifuging it for 10 minutes. The serum was injected intravenously into the femoral vein using a No. 27 gauge needle. All injections were made immediately after the serum had been collected. The donor-recipient plan of Table I was followed on the day of irradiation, the day following, and 4 days after the irradiation of the rats. The rats injected on the day following and the 4th day after the irradiation received an average dose of 0.45 cc of serum/100 g body wt, while those injected on the day of the irradiation received 0.34 cc of serum/100 g body wt.

The recipient rats were observed for a period of 30 days. No deaths occurred in any of the experimental groups as a result of the injections. The body weight - time variation of corresponding groups did not show any significant differences. Therefore, the reported toxic effect of the irradiated serum was not amplified by preliminary sensitization of the recipients.

In another experiment, 10 rats were injected intraperitoneally with 1.0 cc of serum of rats treated 24 hours previously with a total body dose of 640 r of

X rays (83 kv, 1 mm of Al, T.S.D. 30 cm tissue dose rate 21 r/min). Five rats were also injected intraperitoneally with 1.0 cc of unirradiated serum. The differential white blood cell count was taken before and 24 hours after the injection. The results are shown in Table II. Percentages for monocytes, eosinophils, and basophils are not reported since they account for such a small percentage. The injection had no effect on the lymphocyte count of the recipients.

TABLE II  
Average percentage of leucocytes

Injection	Time after the injection, hours	Lymphocytes, %	Neutrophils, %
Normal serum	0	74	23
Normal serum	24	78	19
Irradiated serum	0	75	24
Irradiated serum	24	76	22

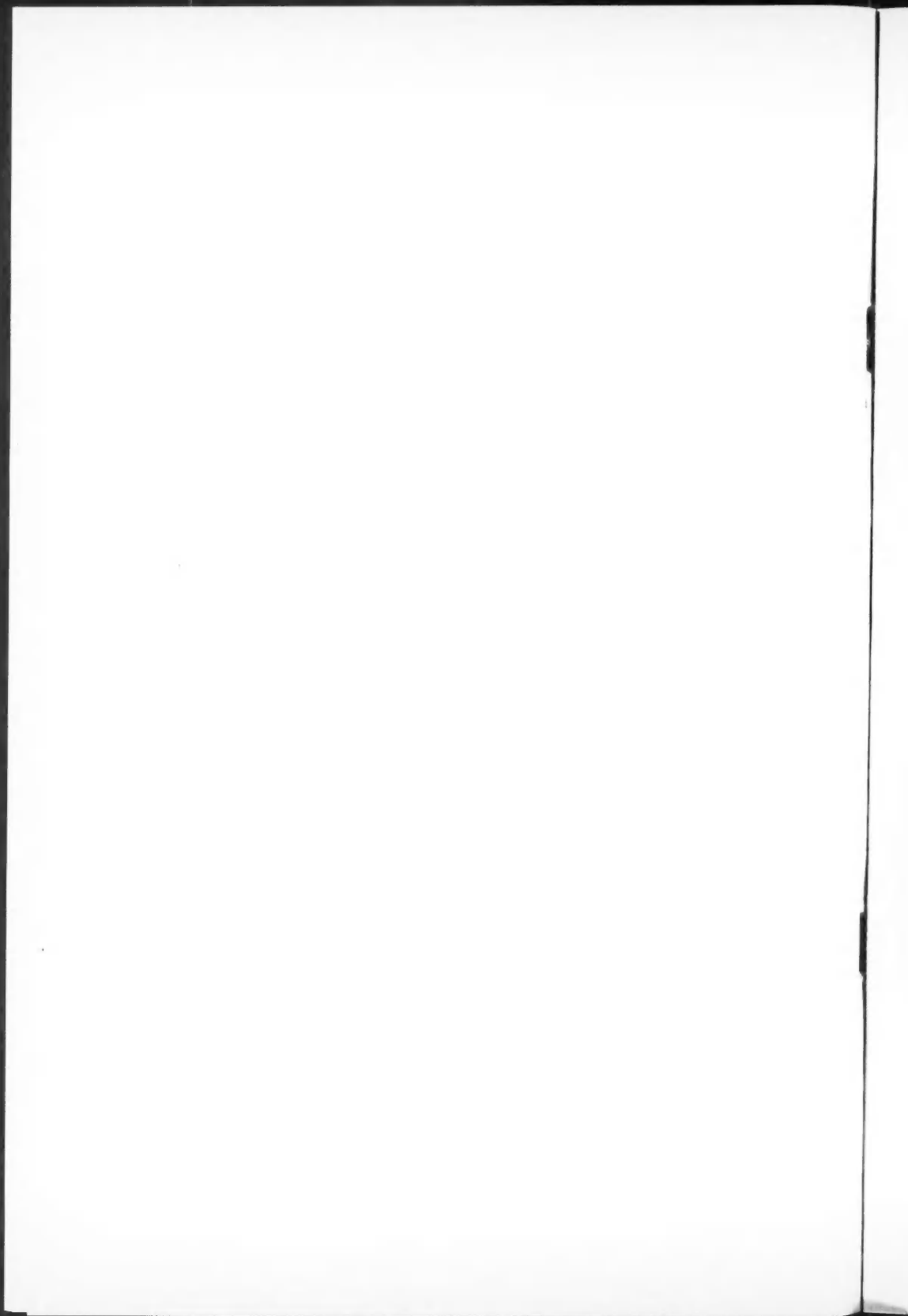
The results of the above experiments, although preliminary in nature, corroborate the negative results recently published by Campo *et al.* (1958(1)).

#### Acknowledgments

The authors wish to thank C. Chaundy of the Physics Section, Commercial Products Division, Atomic Energy of Canada Ltd., who performed the  $\text{Co}^{60}$  irradiations and measured the doses. The authors are also grateful to E. R. W. Gregory for helpful advice and assistance in handling the animals and to Jacqueline Leonard for technical assistance.

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### *Canadian Journal of Zoology*

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